Vitreous State Laboratory

The Catholic University of America

Washington, D.C. 20064

Mechanisms of Microwave Induced Damage in Biologic Materials

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by

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In an effort to establish a laboratory basis for the indications from epidemiological studies of adverse health consequences of extremely low frequency (ELF) electromagnetic field (EMF) exposure, researchers around the world have investigated the teratogenic potential of weak EMFs on developing chick embryos. These results have been contradictory, and thus inconclusive. Even in those studies where a robust effect has been found, unexpected variations with field strength and exposure intervals have cast doubt on the data. This report addresses both the questions of experimental repeatability and of the unusual dose/response behavior. The difficulties in replication are attributed to a genetic predisposition of the embryos combined with the presence of prior stresses. The problem of field strength and exposure

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interval dependence is tackled by suggesting that a previously described multi-step chemical reaction model can be employed. Data from our laboratory and others are offered in support of these hypotheses.

Exposure of L929 cells to either 60-Hz amplitude-modulated microwaves, or to 60-Hz electromagnetic fields, produced an approximately twofold transient enhancement of ornithine decarboxylase (ODC) specific activity. The requirement for signal coherence to achieve ODC enhancement was investigated by switching the ELF or amplitude modulation signal from 55 to 65 Hz at selected intervals. Results showed that for either system a coherence time of 1.0 s or less resulted in no enhancement, but that coherence times of 10.0 s or more produced full enhancement.

The effect of an applied 60-Hz, 1-mT magnetic field upon processing rate of the 45S pre-rRNA molecule in HL-60 cells was investigated with pulse-chase techniques. Results showed that the rate of processing of the 45S molecule to form 18S and 28S rRNAs was accelerated in the field. Additional work examined the rate of synthesis of specific mRNAs in the 60 Hz field. This was done using an S_1 nuclease protection assay, devised for the project as a means of replacing the standard nuclear run on technique. Results showed electromagnetic field enhancement of the synthesis of 45S pre-rRNA, but no alteration of the rates for either c-myc or actin mRNAs.

SAR measurements in a newly installed IFI BC 110 Crawford cell based system indicated considerable non-uniformity of the electric field distribution in the region of interest within the sample flasks. Because of constraints due to available sample containers and cell population requirements, the necessary conditions to improve the electric field distribution have not been met with this Crawford cell. Results of our studies of the distribution of the induced electric field within homogeneous saline samples exposed to ELF magnetic fields show that with certain geometries it is possible to achieve essentially uniform induced electric fields within the region of interest. Additional studies using two layer systems modeling a settled cell suspension have shown that these results are also applicable to this latter case. A computer controlled signaling scheme was devised to investigate the effect of the coherence time of the exposure signal on ELF biological effects. This scheme was used to operate both the "Henhouse" exposure system and a microwave exposure system.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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CHAPTER 1

Electromagnetic-field-induced teratogenesis in chicken embryos:
Genetic predisposition and power/time windows

1.1. INTRODUCTION

The past decade-and-a-half has witnessed the accumulation of epidemiological evidence suggesting a connection between a variety of adverse effects on human health and exposure to low-level extremely low frequency (ELF) electromagnetic fields (EMF). In an effort to establish a laboratory basis for these epidemiological indications, researchers around the world have investigated the teratogenic potential of weak EMFs on developing chick embryos. These results have been contradictory, and thus inconclusive. Even in those studies where a robust effect has been found, unexpected dependences on field strength and exposure intervals have cast doubt on the data. Our research during the past year has addressed both the question of experimental repeatability and the problem of field strength and exposure interval dependence. In the former case our work has led us to propose that the difficulties in replication are connected with a genetic predisposition of the embryos combined with the presence of prior stresses. Data from our laboratory and others are offered in support of this hypothesis. The problem of explaining the dependence of the teratogenic response on the strength and timing of the exogenous field is tackled by suggesting that a previously described multi-step chemical reaction model (Litovitz *et al.*, 1990) can be employed. It is shown that both the maximum in teratogenic sensitivity as a function of field strength and the dependence of the teratogenic effect on the exposure interval can be understood in terms of this model.

1.2. BACKGROUND

Over the past 11 years a series of epidemiological studies have found an increased risk of cancer among children who live close to power lines (Werthheimer and Leeper, 1979; Savitz, 1988; Lin, 1989). The research indicates that children living in homes experiencing high ambient electromagnetic fields have a 50% greater risk of developing cancer, particularly leukemia, lymphomas, and nervous system tumors. In a recent study in the Los Angeles area Preston-Martin and Mack (1990) at the University of Southern California found that men who had worked for 10 years or more in a variety of electrical occupations had a tenfold increase in brain cancer incidence relative to the control group. Other data also show that workers in occupations requiring electromagnetic field exposure, such as electricians and power and telephone linemen are at higher risk for brain tumors and other cancers (Milham, 1976, 1982, 1983, 1985).

A study performed by Matanoski *et al.* (1989) found a dose response relationship for cancers in males employed by New York Telephone from 1976 to 1980. Matanoski measured the average magnetic field exposure among different types of employees and found that cable splicers had by far the largest doses, followed by central office employees and then installation and repair workers. A comparison of the cancer rates among the various types of employees showed that cable splicers were nearly twice as likely to develop cancer as those employees who did not work on telephone lines. Among central office workers (who are exposed to the short intense fields of telephone switching equipment), the rates of several cancers were unusually high, although not as high as for cable splicers. The central office workers were more than three times as likely to get prostate cancer and more than twice as likely to get oral cancer as co-workers who were less exposed. In addition, there were two cases of male breast cancer, the normal incidence of which is so rare that no cases at all would be expected.

Another important set of experiments involves the effect of electromagnetic fields on the melatonin production of the pineal gland. Melatonin is a regulatory hormone whose levels have been linked to several cancers, especially breast and prostate, as well as to the functioning of the immune system. Studies by Wilson (1981) have shown that 60 Hz fields can reduce the amount of melatonin in a rat's pineal gland at night when these levels are normally at their peak. Researchers have found that lower melatonin levels leave rats vulnerable to chemically induced mammary tumors.

Establishing a cause-and-effect confirmation of low frequency electromagnetic fields with adverse health effects such as are suggested by these epidemiological correlations has proven to be elusive. *In vitro* experiments have revealed the existence of a number of field-induced effects at the molecular and cellular level at field strengths (0.1 μT to 10 μT) comparable to ambient values in residential settings. Goodman *et al.* (1990) have shown that transcription can be augmented by fields of this magnitude, finding, for example that the mRNA concentrations of the myc oncogene can be enhanced by factors of four or more. Others have shown that the activity of important enzymes can be enhanced by these weak magnetic fields (Krause *et al.*, 1990). Although it is clear that weak electromagnetic fields can cause cellular responses, there has been no unambiguous connection of these to deleterious health consequences in humans.

On the other hand data have been reported showing a strong connection between low level fields and abnormalities in developing chick embryos. However inconsistent results in attempting to replicate these data has led to considerable controversy over whether or not the effect really exists. Below we consider the cause of this difficulty in replication. It will be explained in terms of a predisposition to the teratogenic effects of EM fields. In addition it will be shown that the existence of "power windows" (i.e. a peak in abnormality rate is observed as function of the field strength) is related to a "time window" of susceptibility of the embryo.

1.3. TERATOGENIC EFFECTS OF EM FIELDS ON CHICK EMBRYOS

Nearly a decade ago Delgado and co-workers (1982) reported that low level, low frequency electromagnetic fields could seriously affect the early development of chick embryos. The idea that these might serve as a model system for investigating the potential health hazards of extremely low frequency (ELF) magnetic fields has stimulated much research during the intervening years. A number of these subsequent chick embryo studies have corroborated the teratogenic effects of 1 to 10 μT magnetic fields (Ubeda *et al.*, 1983; Leal *et al.* (1984); Juutilainen *et al.*, 1986; Juutilainen and Saali, 1986; Juutilainen, Läära and Saali, 1987; Martin, 1988 and 1990).

However, other investigators have not been able to replicate this sensitivity of chick embryos to electromagnetic fields during their early development (Maffeo, Miller and Carstensen, 1984; Maffeo *et al.*, 1988; Sisken *et al.*, 1986; Sandstrom, Mild and Løvtrup, 1986). Consequently, considerable controversy has evolved regarding the teratogenic potential of low level magnetic fields. To resolve the issue the EPA conducted a series of parallel experiments under carefully specified conditions--the so-called "Henhouse" experiment (Berman *et al.*, 1990). Six different laboratories using duplicate equipment and identical exposure conditions were used. Despite meticulous efforts to eliminate experimental differences between the laboratories, there were significant differences in the results. Out of six laboratories, two found significant increases in abnormalities following exposure, two found a small but not statistically significant increase, and two found no effect at all.

We are left with the perplexing question of why some laboratories obtain robust results demonstrating sensitivity of the developing embryo to electromagnetic fields, yet other labs demonstrate no effect at all. Moreover, even in those experiments where teratogenic effects were consistently observed, "windows" of sensitivity to specific field strengths and exposure interval were noted. In this paper we develop a unified picture that allows us to understand these many seemingly contradictory experimental results.

1.3.1. GENETIC PREDISPOSITION FOR TERATOGENESIS IN CHICK EMBRYOS

1.3.1.1. EXPERIMENTAL METHODS

In order to explore this disparity we replicated the "Henhouse" experiment in our laboratory. Our experimental techniques duplicated those of the "Henhouse" experiment (Berman *et al.*, 1990). In fact, two of our exposure systems were actually used in one of the six "Henhouse laboratories" (EPA, Maryland site). Two additional exact replicas were built. Fertilized White Leghorn eggs were obtained from Truslow Farms of Chestertown, Maryland. Just as in the "Henhouse" investigations, we used unipolar, pulsed, magnetic fields (500μs pulse duration, 100 pulses per second, 1μT peak intensity, with a 2 μs rise time and fall time). These pulses were applied to the experimental eggs during the first 48 hours of incubation. Simultaneously an equal number of control eggs (10 in each run) were sham-exposed. After the

48 hour incubation, embryos were removed from their shells and examined histologically by procedures described by Berman (1990). This evaluation was performed under blind conditions.

1.3.1.2. RESULTS

As shown in the first line in Table 1.1 our first experiments (February-April 1990) indicated a robust effect. A statistically significant increase in the number of abnormal embryos was observed when the "Henhouse" exposure protocols were applied. This increase agreed well with the two "Henhouse" laboratories which demonstrated significant increases. However, when the experiments were repeated in September-November 1990, these initial results could not be replicated. The electromagnetic fields had no statistically significant effect.

The major difference noted between the early-1990 and late-1990 runs was the number of abnormalities observed in the control eggs. For the later runs, abnormality rates in the unexposed embryos averaged 20% compared with about 10% for the earlier studies. Extensive testing for stray magnetic fields in the laboratory showed no difference when compared to the ambient fields measured in February through April 1990. We then discovered that other users of eggs from our suppliers were also experiencing high abnormality rates in their controls. In December 1990 a new young flock was put into production; with this flock, the teratogenic sensitivity of the embryos to low level EM fields reappeared. Remarkably, also at this time eggs from the initial flock (that used from February through November) began again to demonstrate a sensitivity to EM fields. It appears that the ability to induce abnormalities by exposure to magnetic fields is related to the abnormality rates that were present in unexposed embryos. The September-November production was obviously subject to greater heat stresses compared to the February-April and December productions, suggesting that heat or some other environmental factor is implicated.

To explore further the effect of the proportion of abnormal controls on the sensitivity of developing chick embryos to EM fields, we reviewed the published literature and compared it with our results. These data are listed in Table 1.1 in order of increasing proportion of abnormal controls. Ignoring temporarily the data from NIOH [Sweden] (these will be considered separately below), an inverse correlation between the fractions of control and field-induced abnormals can be seen. The laboratories which reported teratogenic effects had similarly low proportions of abnormal controls (below 15%), while the laboratories reporting no effects from electromagnetic field exposure had high rates of abnormal embryos in the controls (above 20%). Also interesting is the observation (still ignoring the NIOH [Sweden] data) that the total number of abnormals in the exposed group was relatively constant from one laboratory to another, ranging from a low of 22% to a high of 35% and averaging about 26%.

The relative constancy of the total number of abnormals in the exposed group is intriguing and highly suggestive. Consequently, we considered other data on the effect of a variety of waveforms on chick embryos obtained in our laboratory over a period of one year. Again the same pattern emerges. As the percentage of abnormal controls increased the percentage of field-

induced abnormals tended to decline. The percentage of abnormals in the exposed groups remained in the range 20 to 35%.

1.3.1.3. DISCUSSION

The disparate results obtained by laboratories around the world on the effect of electromagnetic fields on chick embryos has generated considerable controversy. Our results suggest that the fields can indeed have a profound effect at least in certain experimental models. In the white leghorn strain, the teratogenic effect of electromagnetic fields *is* reproducible whenever the proportion of abnormal controls is low.

The first important observation is the appearance of an apparent "ceiling" to the abnormality rate in exposed embryos. The chick embryo data reported in Table 1.1 support the hypothesis of a susceptible population, as no investigators were able to produce abnormalities in more than 35% to 40% of chicks. That this is not simply a dose-limited effect is confirmed by experiments utilizing field magnitudes up to 100 µT. In both Juutilainen's work (1987) and our own, these elevated field levels were unable to induce abnormality rates in excess of about 40%. The observation of an upper limit in the rate of occurrence of abnormalities points to a genetic difference in susceptibility to electromagnetic fields within the chick embryo; only susceptible embryos can manifest the abnormality.

This first conclusion is based on known principles of teratology and observed differences in human susceptibility to teratogens. Even with one of the most potent human teratogens known, thalidomide, exposure of the embryo during the critical period of gestation resulted in limb reduction abnormalities in only 20% of embryos (Lenz and Knapp, 1962; Knapp, Lenz and Nowack, 1962). A review of drugs and chemicals proven to be teratogenic in humans demonstrates that abnormalities occur at rates less than 40%, and usually much lower, even when these potent teratogens are considered (Table 1.2). Higher abnormality risk rates appearing in the teratology literature are limited to drugs and chemicals for which the frequency of malformations is calculated from case reports. In these instances, inherent biases of spontaneous reporting lead to overestimates of abnormality rates, as normal outcomes are less often reported.

The observation of a maximum malformation risk following exposure to a teratogen suggests that individual embryos differ in susceptibility to the teratogen. The teratogen alone does not determine whether an embryo will be normal or affected. Recent developments indicate that this susceptibility is genetic, with malformations resulting from the interaction of the genotype and environmental factors. Thus differences in teratogenesis may be considered a genetic trait, with susceptibility or liability genes in either the maternal or fetal genotype (Finnell and Chernoff, 1987). Mutations, or liability genes, may alter the metabolic fate of a given drug or chemical, leading to deranged embryonic and fetal development.

Evidence supporting the role of genetic factors is provided by studies of the metabolism and teratogenicity of the anticonvulsant drug phenytoin. Phenytoin is metabolized to highly reactive arene oxide intermediates which are capable of covalent binding to embryonic or fetal

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macromolecules and nucleic acids, disrupting normal development. Individuals with low levels of epoxide hydrolase may accumulate toxic arene oxides, increasing their susceptibility to the toxic effects of epoxides. Measurement of fetal amniocyte epoxide hydrolase activity allows *prenatal* prediction of affected offspring, and provides confirmation that the teratogenicity of phenytoin is mediated, at least in part, by toxic intermediates produced during the biotransformation of phenytoin (Buehler *et al.*, 1990; Koren, 1990).

A gene-teratogen interaction has also been identified with a non-chemical environmental stress. Inbred mice strains exhibit differing frequencies of heat-induced exencephaly, indicating a genetic component of susceptibility (Finnell and Chernoff, 1987).

Although the biochemical mechanism or genetic locus of susceptibility has *not* been elucidated for most teratogens, the theory that teratogens act on susceptible genotypes to cause birth defects is now well accepted. Therefore, we feel it is appropriate to apply these principles to the EMF data gathered in exposed chick embryos in our laboratory and the laboratories of others. However, we still must explain the consistent failure to demonstrate an EMF effect in chick embryos with high rates of abnormalities among controls. Here we hypothesize that a prior environmental stress (possibly heat, since the high abnormal rates began in August) has acted upon the genotype of the chick embryo. We can exclude the possibility that this prior environmental stress was actually an unidentified pre-incubation EMF exposure based on the known failure to observe abnormalities in chick embryos exposed prior to 12 hours incubation (Martin, 1990). We must further hypothesize that the prior environmental stress interacts with the genotype in a manner similar to the EM interaction. Perhaps both the EM stress and the unidentified environmental stress act on a related genetic locus; or perhaps the unidentified environmental stress regulates the expression of this gene.

Our unifying theory must also explain the apparent deviation of the NIOH [Sweden] results from the high-control-abnormal/low-induced-abnormal correlation noted for other results. We think it is significant that this is the only one of the laboratories participating in "Henhouse" that did not utilize eggs obtained from a large commercial flock. We hypothesize that the small Swedish flock was less genetically diverse than commercial flocks, and was characterized by a lower inherent genetic susceptibility rate than those flocks.

We conclude that in the appropriate experimental model, namely, white leghorn chick embryos with a low intrinsic incidence of abnormalities, low amplitude ELF electromagnetic fields induce additional abnormalities in these embryos. Not all embryos are susceptible to EM-induced abnormalities, just as not all humans have the same risk of abnormal offspring following exposure to known teratogens. These differing susceptibilities are likely based on a genetic predisposition; we propose that prior environmental stresses, such as heat, may mask the field-induced effect in the experimental model.

1.3.2. TIME AND POWER WINDOWS IN CHICK EMBRYO TERATOGENESIS

The developmental changes in chick embryos exposed to extremely low frequency electromagnetic fields indicate the existence of domains of enhanced sensitivity to both the intensity of the field (power windows) as well as to the time interval during which the field is applied (time windows). In this section we show that an extension of the linear multi-step chemical model described elsewhere (Litovitz *et al.*, 1990, Montrose, Litovitz and Elson, 1991) accounts for these features of the data.

The general plan of this section is as follows. First, we organize the experimental results (which are taken from previously published work) to be examined, and pose the specific questions suggested by these data. Following that, the mathematical model is summarized and the modifications necessary for applying it in this context are indicated. Finally the data are interpreted within the framework of the model and the answers to the questions developed from the results are suggested.

### 1.3.2.1. THE RELEVANT EXPERIMENTAL DATA--POWER AND TIME WINDOWS

The data of Juutilainen and co-workers (1986, 1987) provide evidence for the existence of a "power window", i.e., the response of the biological system exhibiting a peak when regarded as a function of the exogenous field strength. The Juutilainen data for 16.7 Hz sinusoidal waveforms are given in Figure 1. The question to be asked is what sort of response characteristics of the developing embryo can lead to the appearance of maximum-sensitivity windows for certain field strengths.

A second feature of the data for EMF-induced changes is exemplified by recent work of Martin (1990) summarized in Table 1.3. The first three entries in the table show that the teratogenic effects are produced by exposure during the first twenty-four hours of incubation. No significant increase is observed in embryological damage by continuing the exposure beyond this period. Moreover, embryos exposed only during the second twenty-four hours of incubation exhibited no increase in abnormal development. These results suggest that there is a time period during the first day of incubation during which embryos are particularly sensitive to the presence of EM fields. The fourth and fifth entries in the table confirm this idea--the critical time period for producing the abnormalities being discussed here is 12 to 24 hours after the start of incubation. What remains to be explained is why, since no increase in abnormal embryos is produced by a 0-to-12 hour exposure, the number of abnormal embryos that result when the field is applied only during the second twelve-hour period is so much less (about half) than that obtained for exposure during the 0-to-48 or 0-to-24 hour intervals.

1.3.2.2. THE MULTI-STEP CHEMICAL MODEL

The model was originally developed by Litovitz et.al. to account for augmented transcription (Goodman, Bassett and Henderson, 1983; Goodman, Abbott and Henderson, 1987; Goodman

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and Henderson, 1988) in cell populations exposed to low frequency pulsed and sinusoidal electromagnetic fields. The basic idea is that transcription is envisioned as a series of reaction-type events described by a set of linear first order differential equations. Schematically

$$[A] \xrightarrow{k_1} [X] \xrightarrow{k_2} [Y] \xrightarrow{k_3} \cdots$$
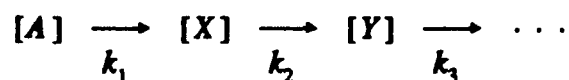
where [A] describes the nucleotide pool, [X], [Y] ... represent a set of reaction products that are synthesized sequentially and the k's are rate constants that describe the speeds at which the reactions progress. The first step (with rate constant $= k_1$) represents the diffusion-controlled migration to, and the "positioning" and orienting of the various nucleotides from the cellular pool in their "correct" sites in the neighborhood of the DNA molecule. The second reaction step (rate constant $= k_2$) is the polymerization of the positioned and oriented nucleotides to form messenger RNA. The final step (rate constant $= k_3$) is the degradation of messenger RNA by cytoplasmic nucleases. The reactions are taken to be strongly biased in the forward direction, so that only these rate constants need be considered. Of course, what is described as a *single* reaction step in actuality consists of a number of complex processes; our assumption is that among these processes there is one that is "rate-determining" so that the simplification shown above is meaningful. No qualitative changes in the model's predictions would result if additional steps were added to the sequence, although the equations would become more complicated and the exact shapes of the response curves would be altered.

For the case of embryological development, the same formal structure can be maintained but the various elements must be reinterpreted: [A] perhaps describes the amino acid pool from which proteins [X], [Y] ... are synthesized. We assume that [Y] corresponds to a particular protein or group of proteins the synthesis of which, *in appropriate concentrations*, is crucial for normal embryological development. While this is certainly oversimplified, it offers the advantage of being mathematically tractable, and can therefore serve as a useful "zeroeth-order" approximation in terms of which the response to EMF exposure can be understood.

The crucial hypothesis of the model is the supposition that the immediate effect of initiating exposure to EMFs is an increase in the rate constant characterizing one of the intermediate reactions. In particular, we suggest that switching on the electromagnetic field at $t = 0$ produces a "sudden" increase in k_2, that is,

$$k_2 \longrightarrow k_2^*,$$

with this change occurring on a time scale that is short compared with the inverse reaction rates.

The mathematical consequences of this are easily derived. Assuming that the reservoir [A] is rapidly replenished and that these reactions can be described by a set of linear first order differential equations gives the time dependent response following the switching on of the field at time $t = 0$ as

$$y(t) = \frac{k_1 a}{k_3} + \frac{k_1 a}{k_3 - k_2^*} \frac{\Delta k}{k_2} [e^{-k_2^* t} - e^{-k_3 t}], \qquad (1.1)$$

In this a and y denote the concentrations of [A] and [Y], respectively and

$$\Delta k = k_2^* - k_2. \qquad (1.2)$$

The bioresponse $y(t)$ is plotted in Figure 2(a) for three values of the altered rate constant k_2^*, the other rate constants (k_1, k_2 and k_3) being the same for each of the curves on the graph.

Observe from Figure 2(a) that, independent of the value of k_2^*, the bioresponse $y(t)$ returns at long times to the basal (equilibrium zero-field) level, i.e., $y(\infty) = y(0)$. The transient character of the response in this model is relatively easy to understand. The initial growth of $y(t)$ arises from the enhanced rate k_2^*; the drop off at long times (back to the equilibrium value) comes from the depletion of the essential precursors. Several features of the dynamical behavior are noteworthy:

    (1)   the bioresponse $y(t)$, peaks earlier with increasing k_2^*, and

    (2)   the peak height increases with increasing k_2^*.

These observations allow one to understand the phenomenon of so-called "power windows", i.e., the apparent enhanced sensitivity of the response to an intermediate dose level, for measurements made at a particular exposure time T. The only assumption that is needed is that k_2^* increases monotonically with the exogenous field strength. Then it is easily seen from Figure 2(a) that measurements made at the time T after switching on the field result in the appearance of a maximum in the response considered as a function of k_2^* (or equivalently field strength or power density). This can be seen in the bar graph in Figure 2(b). The value of $y(T)$ is more than 30% greater for the intermediate value of k_2^* (field strength) than it is for either the larger or smaller value. It is important to note that this peaking of the response as a function of the change in k_2 (or field strength) is not an accident of the choice of measurement time; it cannot be made to vanish by changing T. A different value of the measurement time will shift the maximum in the response to a different value of Δk; the longer the measurement time, the smaller is the value of Δk at which the response peaks. This is not apparent from Figure 2(b) which is a simplified rendition in which curves for only three values of Δk are shown.

Equation (1.1) for the biological response to the applied field describes the case in which the field is switched on at time = 0 and allowed to remain on throughout the experiment. The response can also be determined for the case in which the field, having been switched on at time = 0, is switched off at some instant of time = t^*. For times $0 \le t \le t^*$, the response is still given by equation (1.1); however, for $t > t^*$ the result is

$$y(t) = y(t^\circ)e^{-k_3(t-t^\circ)} + \frac{k_1 a}{k_3}[1 - e^{-k_3(t-t^\circ)}]$$

$$+\frac{k_1 a}{k_3 - k_2}\frac{\Delta k}{k_2^*}[e^{-k_3(t-t^\circ)} - e^{-k_2(t-t^\circ)}],$$

(1.3)

where we have assumed that the rate constant k_2 returns to its original value when the field is switched off, i.e., $k_2^* \to k_2$.

1.3.2.3. APPLICATION OF THE MODEL TO THE CHICK EMBRYO DATA

The multi-step chemical model is able to suggest answers to two questions raised by the chick embryo exposure results:

(1)   What sort of response characteristics of the developing embryo can lead to the appearance of maximum-sensitivity windows for certain field strengths? and

(2)   Since no increase in abnormal embryos is produced by a 0-to-12 hour exposure, why is the number of abnormal embryos that result when the field is applied only during the second twelve-hour period considerably fewer than that obtained for exposure during the 0-to-24 hour interval?

The answers to both of these questions involve recognizing that there is a *relatively narrow time interval* in embryomic development during which EMF exposure causes teratogenic effects. During this interval corresponding to a period of cell differentiation the threshold for teratogenesis (that a threshold level exists can be inferred from Juutilainen's (1987) data) is considerably lower than at other times in the development. This essentially fixes a measurement time T--actually a time range, say T to $T + \Delta T$--that allows the power-window feature of the chemical model to be applied to chick abnormalities. Figure 3 illustrates the point. We have assumed that the period 16-to-22-hours after the start of incubation (roughly Hamburger and Hamilton's (1951) stages 3 and 4) corresponds to the window of maximum sensitivity. This is indicated on the graph by a depression in the plot of teratogenic threshold. The shaded region emphasizes the extent to which the intermediate-Δk curve exceeds the threshold. The parameters used to compute the bioresponse, defined here as

$$\Delta y(t) = y(t) - y_{eq},$$

(1.4)

are $k_1 = 1/60$ h^{-1}, $k_2 = 1/60$ h^{-1} and $k_3 = 1/6$ h^{-1}, and the values of k_2^* corresponding to the three response curves are 1/30, 1/10 and 1 h^{-1}. Because the system's response $y(t)$ is proportional to a, we have chosen a as the basic concentration unit, i.e., $a = 1$. Equivalently, we may regard the response as being given by a ratio with a, i.e., $y \rightarrow y/a$. From the figure it is clear that the high- and low-field response curves never exceed this threshold, whereas the response for the intermediate field exceeds the threshold for the entire sensitivity interval.

Similarly, it is possible to understand and rationalize the data of Martin presented in Table 1.3. The observation that 24- and 48- hour exposures induce the same level of teratogenesis while a 12-hour exposure produces no embryological change can be understood from the curves in Figure 4(a). These are calculated from Eqs. (1.3) and (1.4) using the values $k_1 = k_2 = 1/60$ h^{-1}, $k_2^* = 1/24$ h^{-1} and $k_3 = 1/10$ h^{-1} for the rate parameters. The interpretation is clear.

The multi-step chemical model also accounts for the effect of delaying the switching on of the field until 12 hours after the start of incubation. The idea is illustrated in Figure 4(b). For the field switched on after a 12 h delay, the threshold for teratogenesis is exceeded both for a shorter time and by a somewhat lesser extent than for the undelayed exposure case; thus a smaller increase in the number of abnormal embryos is to be expected. This is reasonable whether one assumes that the probability for embryological alteration is proportional simply to the time interval for which the threshold is exceeded, or if there is also a dependence on the extent to which the threshold is exceeded.

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1.4 TABLES

Table 1.1. Effect of EM pulses on Chick Embryos.
Comparison of Results in Various Laboratories

Lab [Location]	Proportion of Abnormal Controls	Proportion of Abnormal Exposed	Proportion of EM-Induced Abnormals[*]
UWO [Canada] [†]	0.06	0.24	0.18
CUA [DC, USA]	0.10	0.25	0.15
FDA [MD, USA] [†]	0.12	0.23	0.11
NIOH [Sweden] [†]	0.13	0.15	0.02
Sisken [NY, USA] [¶‡]	0.14	0.24	0.10
Maffeo [NY, USA] [§]	0.21	0.22	0.01
UNC [NC, USA] [†]	0.24	0.22	-0.02
Cajal [Spain] [†]	0.27	0.31	0.04
EPA [NV] [†]	0.30	0.35	0.05

[*] Proportion of EM-Induced Abnormals = Proportion of abnormal exposed embryos - Proportion of abnormal controls

[†] Berman *et al.* (1990)

[¶] Sisken *et al.* (1986)

[‡] Berman, E. (1990)

[§] Maffeo *et al.* (1984) and (1988)

Table 1.2. Summary of Teratogenic Risks in Humans
for Selected Proven Teratogens. Data from Koren (1990)

Drug/chemical	Frequency of Malformations Following Exposure of Embryo or Fetus During Susceptible Developmental Stage (% of Those Exposed)
Coumadins	16% malformations; 8% stillbirths
Diethylstilbesterol	39% congenital changes in vaginal epithelium
PCBs	4-20% (with verified high consumption in rice oil)
Phenytoin	5-10% have typical syndrome; 30% have some manifestations
Retinoic acid	38% (predominantly central nervous system effects)
Thalidomide	20% (limb reduction abnormalities)

Table 1.3. Effect of EM field on chick embryos as a function
of the exposure time. Data from Martin (1990).

Period of exposure (h)					Proportion of Abnormal Controls	Proportion of Abnormal Exposed	Proportion of EM Induced Abnormals*
0	12	24	36	48			
//					0.06	0.23	0.17
/////////////////////////					0.06	0.24	0.18
		//////////////////////////			0.14	0.11	-0.03
///////////					0.14	0.13	-0.01
	/////////////				0.07	0.17	0.10

///////// REPRESENTS EXPOSURE TIMES /////////
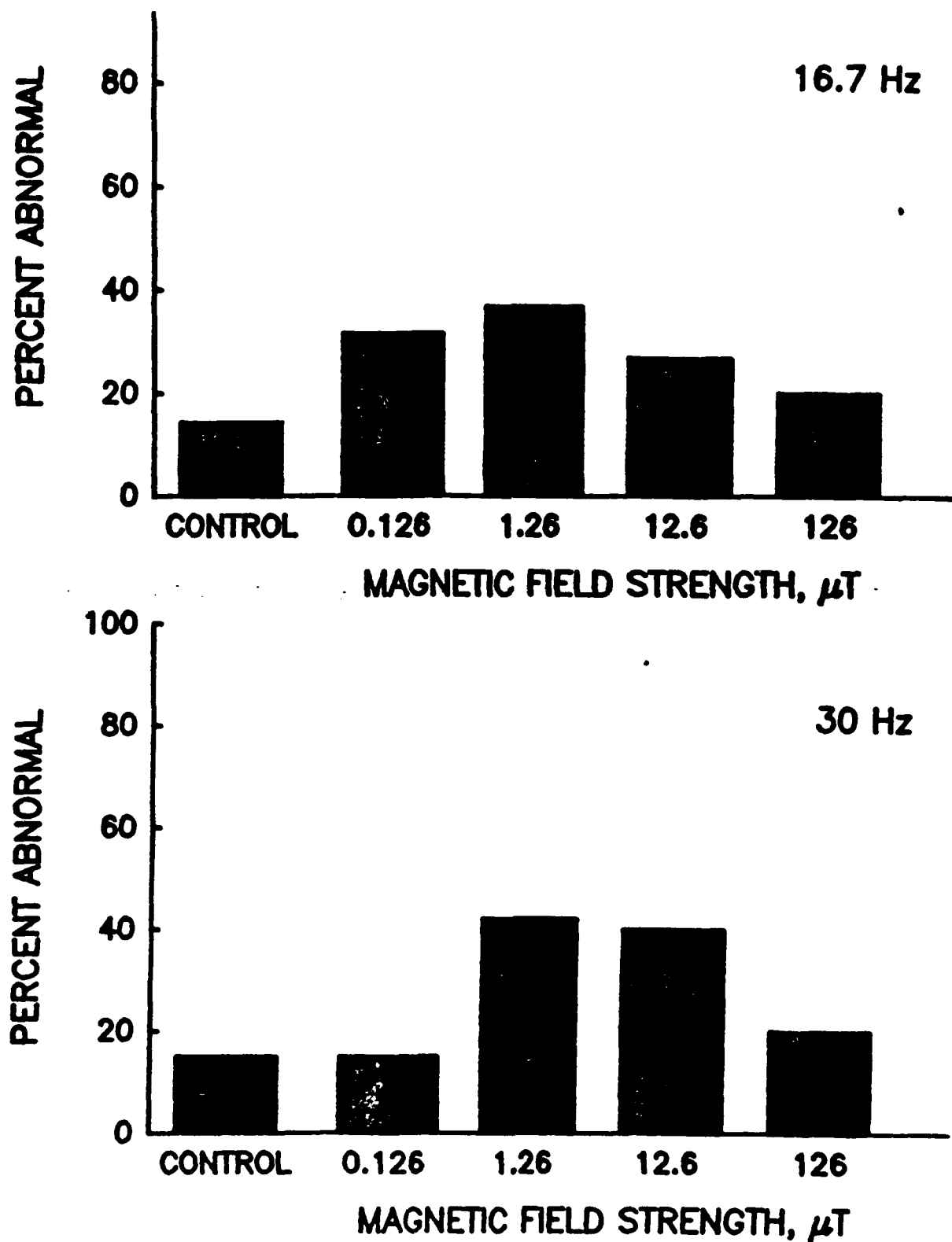
15

Fig. 1.1. The percentage of abnormal embryos observed under various magnetic field intensities for two frequencies. The data are from Juutilainen (1986).
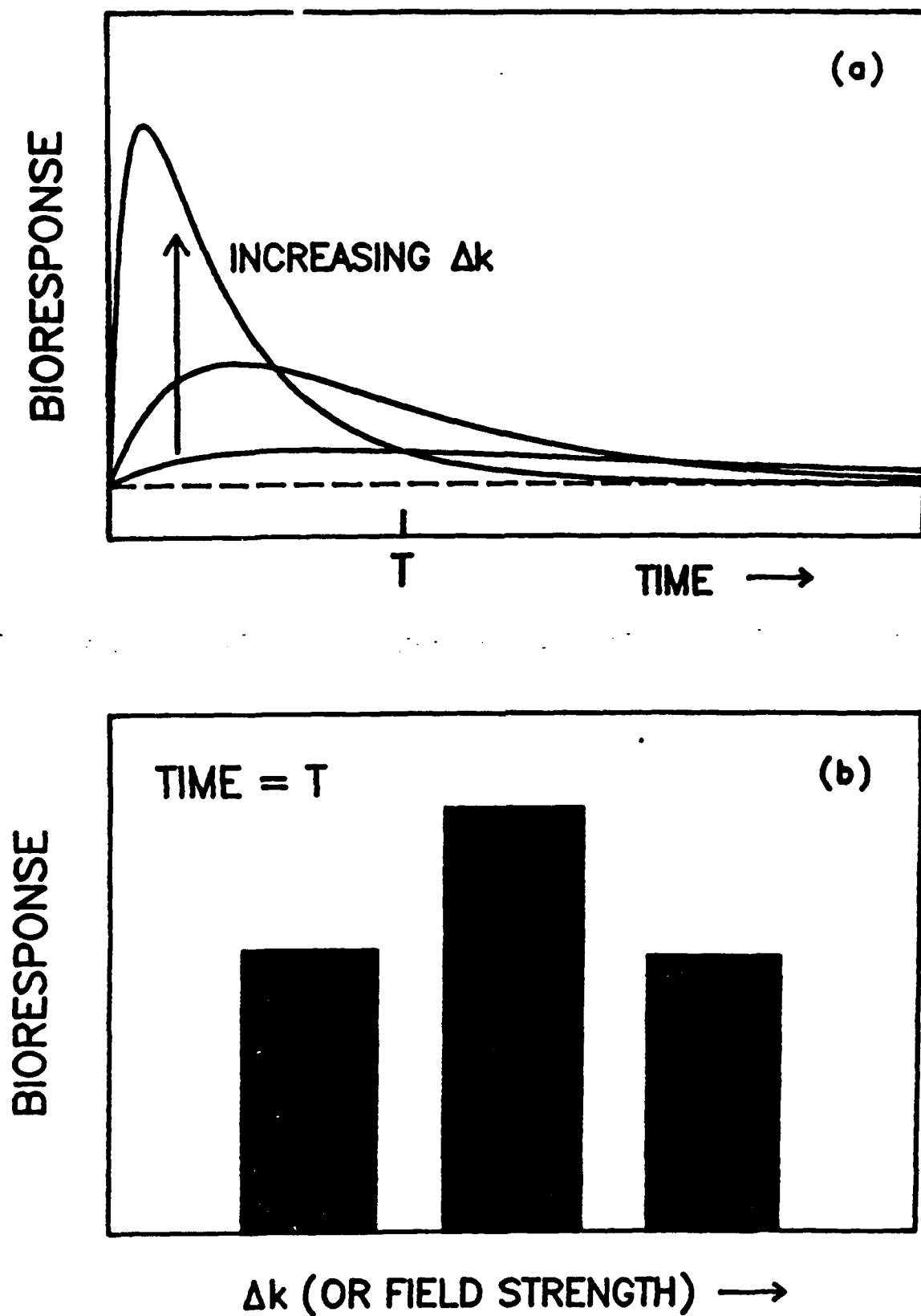
Fig. 1.2. (a) The prediction of the multi-step model for the time evolution of the concentration y(t) following the switching on of the exogenous electromagnetic field at time = 0. The curves are calculated from equation (1.4) for three values of Δk. (b) The apparent "power window" that results when measurements are made as a function of field strength (here characterized by increasing Δk) at one specific time, *T* in (a), after exposure is initiated.
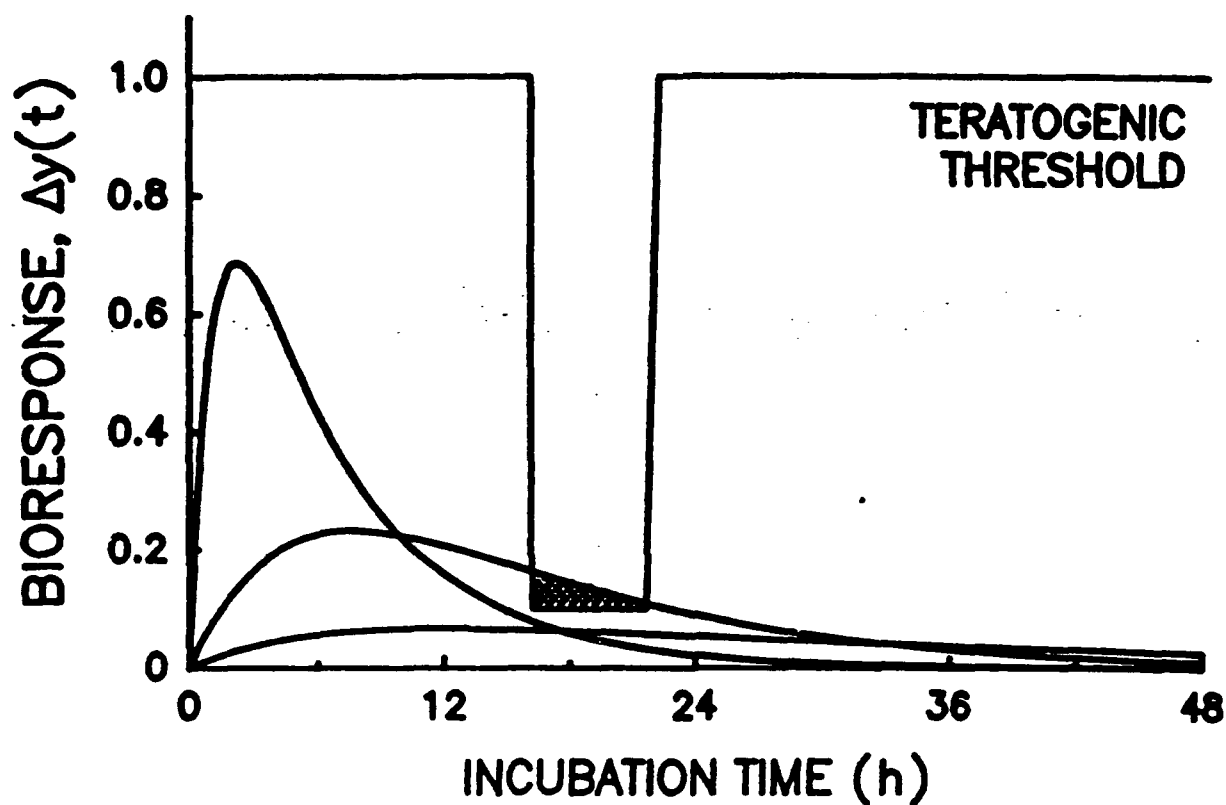
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Fig. 1.3. The bioresponse $\Delta y(t)$ following the switching on of the exogenous electromagnetic field at time = 0. The curves are calculated from equation (1.3) for three values of k_2^*, namely 1/30, 1/10 and 1 h^{-1}. The other rate constants used are $k_1 = 1/60$ h^{-1}, $k_2 = 1/60$ h^{-1} and $k_3 = 1/6$ h^{-1}. A high- sensitivity "time window" is indicated schematically by a time dependent threshold for teratogenesis.
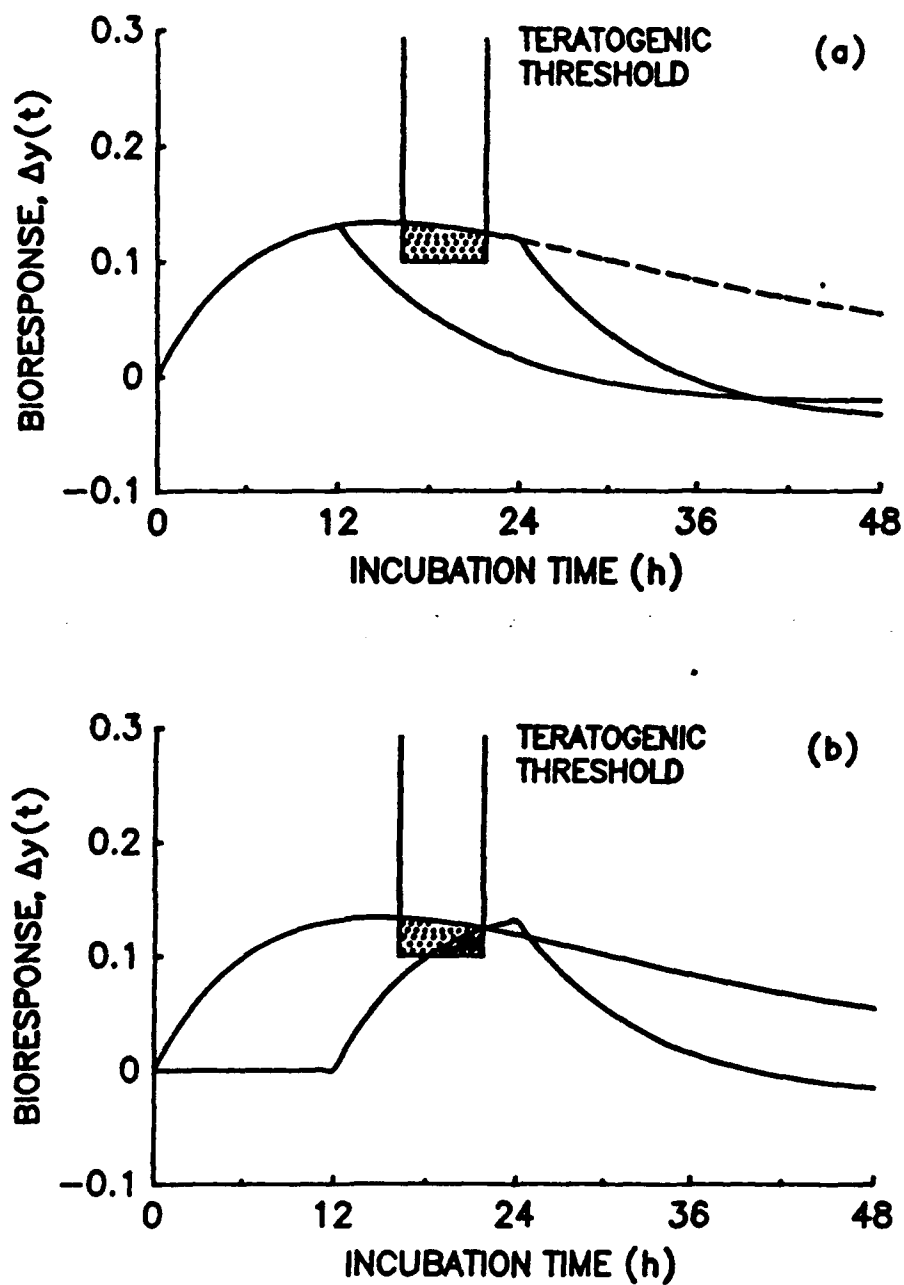
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Fig. 1.4. (a) The bioresponse $\Delta y(t)$ following the switching on of the electromagnetic field at time = 0. Three cases are shown: the field is on for the entire 0 - 48 h period (dashed curve); the field is on for the periods 0 - 12 h and 0 - 24 h. (b) $\Delta y(t)$ for the cases of 0 - 48 h and 12 - 24 h exposures.

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1.6. REFERENCES

Berman, E. (1990) Reproductive Toxicology, *4*:45.

Berman, E., L. Chacon, D. House, B. A. Koch, W. E. Koch, J. Leal, S. Løvtrup, E. Mantiply, A. H. Martin, G. I. Martucci, K. H. Mild, J. C. Monahan, M. Sandström, K. Shamsaifar, R. Tell, M. A. Trillo, A. Ubeda and P. Wagner (1990) The effects of a pulsed magnetic field on chick embryos. Bioelectromagnetics, *11*:169-187.

Buehler, B. A., D. Delimont, M. V. Waes and R. H. Finnell (1990) New Engl. J. Med. *322*:1567.

Delgado, J. M. R., J. Leal, J. L. Monteagudo and M. G. Gracia (1982) Embryological changes induced by weak, extremely low frequency electromagnetic fields. J. Anat., *134*:533-551.

Finnell, R. H. and G. F. Chernoff, in *Pharmacokinetics in Teratogenesis. Volume II Experimental Aspects In Vivo and In Vitro*, ed. H. Nau and W. J. Scott (CRC Press, Boca Raton, 1987), chap 9.

Goodman, R., J. Abbott and A. S. Henderson (1987) Transcriptional patterns in the x chromosome of *Sciara coprophila* following exposure to magnetic fields. Bioelectromagnetics, *8*:1-7.

Goodman, R., C. A. L. Bassett and A. S. Henderson (1983) Pulsing electromagnetic fields induce cellular transcription. Science, *220*:1283-1285.

Goodman, R. and A. S. Henderson (1986) Sine waves enhance cellular transcription. Bioelectromagnetics, *7*:23-29.

Goodman, R. and A. S. Henderson (1988) Exposure of salivary gland cells to low-frequency electromagnetic fields alters polypeptide synthesis. Proc. Nat. Acad. Sci. USA, *85*:3928-3932.

Goodman, R., L-X. Wei, J-C. Xu and A. Henderson (1989) Exposure of human cells to low-frequency fields results in quantitative changes in transcripts. Biochemica et Biophysica Acta, *1009*:216-220.

Hamburger, V. and H. L. Hamilton (1951) A series of normal stages in the development of the chick embryo. J. Morphol., *88*:49-92.

Juutilainen, J., M. Harri, K. Saali and T. Lahtinen (1986) Effects of 100-Hz magnetic fields with various waveforms on the development of chick embryos. Radiat. Environ. Biophys., *25*:65-74.

Juutilainen, J., E. Läära and K. Saali (1987) Relationship between field strength and abnormal developments in chick embryos exposed to 50 Hz magnetic fields. J. Radiat. Biol., *52*:787-793.

Juutilainen, J. and K. Saali (1986) Development of chick embryos in 1 Hz to 1 kHz magnetic fields. Radiat. Environ. Biophys., 25:135-140.

Knapp, K., W. Lenz and E. Nowack (1962) Lancet, 2:725.

Koren, G. (1990) *Maternal-fetal toxicology: a clinician's guide* (Marcel Dekker, New York), chapter 2.

Krause, D., J. A. Brent, J. M. Mullins (1990) Enhancement of ornithine decarboxylase activity in L929 cells by amplitude modulated microwaves. Paper presented at the Twelfth Annual Meeting of the Bioelectromagnetic Society, San Antonio, TX. June 1990.

Lenz, W. and K. Knapp (1962) Arch. Environ. Health 5:100.

Lin, R. (1989) Abstract of presentation at the Annual Review of Research on Biological Effects of 50- and 60-Hz Electric and Magnetic Fields (DOE/EPRI Contractors Meeting), Portland, Oregon, 1989.

Litovitz, T. A., C. J. Montrose, R. Goodman and E. C. Elson (1990) Amplitude windows and transiently augmented transcription from exposure to electromagnetic fields. Bioelectromagnetics, 11:297-312.

Maffeo, S., A. A. Brayman, M. W. Miler, E. L. Carstensen, V. Ciaravino and C. Cox (1988) Weak low frequency electromagnetic fields and chick embryogenesis: Failure to reproduce positive findings. J. Anat., 157:101-104.

Maffeo, S., M. W. Miller and E. L. Carstensen (1984) Lack of effect of weak low frequency electromagnetic fields on chick embryogenesis. J. Anat., 139:613-618.

Martin, A. H. (1988) Magnetic fields and time dependent effects on development. Bioelectromagnetics, 9:393-396.

Martin, A. H. (1990) ELF, PEMF's and development: why the controversy? Proceedings of the XXIII General Assembly of URSI, Prague, Czechoslovakia, August 28 - September 5, 1990, to be published.

Matanoski, G., E. Elliot and P. Breysse (1989) Poster presented at the Annual Review of Research on Biological Effects of 50- and 60-Hz Electric and Magnetic Fields (DOE/EPRI Contractors Meeting), Portland, Oregon, 1989.

Milham, S., Jr. (1976) Occupational mortality in Washington State, 1950-1971. DHEW, National Institute for Occupational Safety and Health, Publication No. (NIOSH) 76-175-A,B,C.

Milham, S. Jr. (1982) Mortality from leukemia in workers exposed to electrical and magnetic fields. New Engl. J. Med. 307:249.

Milham, S., Jr. (1983) Occupational mortality in Washington State, 1950-1979. DHEW, National Institute for Occupational Safety and Health, Publication No. (NIOSH) 93-116.

Milham, S. Jr. (1985) Mortality in workers exposed to electromagnetic fields. Env. Health Persp. *62*:297-300.

Montrose, C. J., T. A. Litovitz and E. C. Elson, (1991) Augmented transcription by electromagnetic field exposure: The experimental techniques affects the measured transient response. Biochemistry and Bioenergetics, in press.

Preston-Martin, S., W. Mack and J. Peters (1990) Astrocytoma risk related to job exposure to electric and magnetic fields. Annual Review of Research on Biological Effects of 50- and 60-Hz Electric and Magnetic Fields (DOE Contractors Meeting), Denver, Colorado, 1990.

Savitz, D. A. *et al.* Case-control study of childhood cancer and exposure to 60-Hertz magnetic fields. Am J. Epidemiol., *128*: 21-38.

Sisken, B. F., I. Fowler, C. Mayaud, J. P. Ryaby and A. A. Pilla (1986) Pulsed electromagnetic fields and normal chick development. J. Bioelectricity, *5*:25-34.

Ubeda, A., J. M. R. Leal, J. L. Monteagudo and M. G. Gracia (1983) Pulse shape of magnetic fields influences chick embryogenesis. J. Anat., *137*:513-536.

Wertheimer, N. and E. Leeper (1979) Electrical wiring configurations and childhood cancer. Am. J. Epidemiol., *109*: 273-284.

Wilson, B. W., L. E. Anderson, D. I. Hilton and R. D. Philips. (1981) Chronic exposure to 60-Hz electric fields: effects on pineal function in the rat. Bioelectromagnetics, *2*:371-380.

CHAPTER 2

Cellular effects of electromagnetic fields

2.1 INTRODUCTION

Work conducted in Year 4 centered on two major topics: 1. Experiments were conducted to examine the effects of microwave exposure on the specific activity of the enzyme ornithine decarboxylase (ODC), to investigate the exposure parameters that determine the ODC response, and to determine the relationship of this response to that produced by exposure to extremely low frequency (ELF) electromagnetic fields; 2. Additional sets of experiments were devised to investigate further the transient enhancement of the 45S rRNA precursor by ELF exposure, and to finish previous work related to ELF effects on transcription of specific genes.

2.2. ENHANCEMENT OF ORNITHINE DECARBOXYLASE SPECIFIC ACTIVITY BY AMPLITUDE-MODULATED MICROWAVES OR 60 Hz ELF FIELDS

Ornithine decarboxylase (ODC), which catalyzes the conversion of ornithine to putrescine, is an essential enzyme in the production of polyamines. Polyamine synthesis is necessary for replication of DNA and, consequently, for cell proliferation (Heby and Persson, 1990; Pegg, 1988). Given ODC's role in essential cell functions, and the fact that ODC is a highly inducible enzyme whose activity is responsive to a wide range of specific ligands (Heby and Persson, 1990), investigators have examined the possibility that exposure of cells to electromagnetic fields might significantly alter the expression of the enzyme. Byus *et al* (1988) demonstrated an enhancement of ODC activity in several mammalian cultured cell lines following a 1-hour exposure to 16-Hz amplitude-modulated 450 MHz microwaves. No effect was observed for unmodulated continuous wave (CW) microwave signals. The enhancements that were seen ranged from 150 to 200% of control values, depending on the cell line used, with the time of maximum activity ranging from 1 to 3 hours following the end of exposure. Enhancement of ODC activity was also demonstrated in response to ELF exposure conditions (Byus *et al*, 1987).

It has, thus, been demonstrated that EMFs can alter ODC activity. For neither AM-microwave nor ELF conditions does the enhancement approach the large increases in activity that are found after serum stimulation of cultures, or by treatment of cultures with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (Byus *et al*, 1988; Heby and Persson, 1990). Nonetheless, EMF exposures produce a significant change in activity, and since alterations in ODC activity correlate with aspects of cell differentiation and of malignant transformation (Byus *et al*, 1988; Pegg, 1988), any change of ODC activity by EMF exposure is potentially of interest. Further, the ODC assay provides a good system for investigation of exposure parameters and for analysis of details of the biological response to a field.

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We have examined alterations in CDC activity in the murine cell line L929 after various conditions of microwave and ELF exposure. Our results, reported here, indicate that 60 Hz, amplitude modulated, 915-Mhz microwaves produce an approximately twofold enhancement of ODC activity, but that continuous wave exposure under the same conditions produces no significant change. We have determined that cells must receive a coherent signal for a minimum period of time in order to respond to the field. Results with ELF studies, paralleling those of the microwave work, indicate that the responses for both AM-microwave and ELF exposure conditions show striking similarities, and that the AM-microwave response is most likely a response to the extremely low frequency of the modulating signal.

2.2.1. METHODS

2.2.1.1. CELL CULTURE

Monolayer cultures of L929 cells (American Type Culture Collection CCL1) were maintained in Eagle's Minimal Essential Medium supplemented with 5% donor calf serum. No antibiotics were added to the medium. For exposures, cultures were prepared the day prior to use, and were seeded so as to be in mid-logarithmic growth by onset of the experiment. Cultures of the human leukemic cell line HL-60 were maintained in the in the same medium, supplemented with 10% fetal bovine serum and sodium pyruvate.

2.2.1.2. MICROWAVE EXPOSURES

Unless otherwise stated, the impressed microwave field was either a continuous wave 915-MHz signal or 60% sinusoidal amplitude modulation of that signal. The amplitude modulation was an ELF sinusoid at the frequencies of 6, 16, 55, 60, 65 or 600 Hz. Specific absorption rate (SAR) for these experiments was 3 mW/g. This level of microwave exposure had previously been determined to produce no appreciable heating of the culture medium (less than $0.1°$ C rise). Four 25 cm^2 flasks of L929 cells were placed into a Crawford Cell for exposure. Four control flasks were placed on a shelf outside the Crawford Cell, positioned so that the cells were at the same height as those inside. The Crawford Cell was maintained in a 37° C cell culture incubator to provide for temperature regulation.

2.2.1.3. ELF EXPOSURES

ELF exposures were conducted using a Helmholz coil system. The coils were positioned vertically, providing a horizontal magnetic field (parallel to the cells' growth surface) and, thus, yielding a relatively uniform induced electric field across the growth surface. Exposures were conducted from 1 to 8 hours, using 60-Hz, sinusoidally varying magnetic fields of 10, 100 or 1000 μT strength. Cells were harvested immediately after exposure, washed, and stored as frozen pellets until processing for ODC activity. One set of experiments was done using HL-60 cells and the solenoid exposure system, (these methods are detailed in Section 2.3 below).

2.2.1.4 ODC ASSAY

ODC activity was determined by a modification of the method of Seely and Pegg (1983). Briefly, the frozen cell pellets were lysed with 100 µl lysis buffer (25 mM Tris-HCl, pH 7.5; 2.5 mM dithiothreitol; 100 µM EDTA; 50 µM pyridoxal 5'-phosphate; 50 µg/ml leupeptin; 0.2% NP-40), vortexed vigorously for 20 sec, and centrifuged at 10,000 x g for 15 min at 4° C to yield S10 supernatants. Protein concentration was determined on the S-10 fractions. 250-µl reaction mixtures (400 µM L-ornithine; 0.125 µCi L-[1-^{14}C] ornithine; 40 µM pyridoxal 5'-phosphate; 1.25 mM dithiothreitol; 30 mM Tris-HCl, ph 7.5) were prepared for each sample, containing 500 µg of S-10 protein added last to initiate the reaction. The reaction was carried out in conical 15-ml glass tubes sealed with rubber stoppers carrying polypropylene wells (Kontes) containing 200 µl, 1.0 N NaOH. Incubations were for 1 h at 37° C in a shaking water bath. The reactions were terminated by the injection of 300 µl 20% TCA, and a further 15-min 37° C incubation driving the generated CO_2 into the NaOH. The NaOH was pipetted from the wells into scintillation vials with 6.0 ml Opti-Fluor scintillation cocktail, and the radioactivity was determined with a scintillation spectrometer. Acid-killed enzyme was used to determine background. Activity units were nM ^{14}C-CO_2 generated/30 min/g protein.

2.2.2. RESULTS

2.2.2.1 MICROWAVE EXPOSURE - CONTINUOUS WAVE VS AMPLITUDE MODULATION

Alterations in ODC activity were compared for both continuous wave and for 60-Hz amplitude modulation of the 915-MHz microwave signal. For both exposure conditions the SAR was maintained at 3 mW/g, for which there was no detectable heating of the culture medium. A time course was conducted for each exposure condition. Cultures were exposed for periods of time ranging from 2 to 24 h, harvested at the end of the exposure period, and washed cell pellets frozen for later processing of ODC specific activity. For purposes of comparison, results of each experiment are expressed as a ratio of the specific activity of the exposed culture to that of its specific control. A ratio of 1.0 would thus indicate that the exposed cells gave an ODC activity identical to that of the control cells. Data for this work are shown in Figure 2.1.

Continuous wave irradiation of L929 cells produced no statistically significant variation from control activities with the exception of cultures harvested after a 6-hour exposure. At this time point exposed cultures showed ODC activities 1.3 times those of the corresponding controls. Eight-hour exposures, however, showed no significant deviations in activity from that of controls. With the exception of the slight rise in activity at 6 h, continuous wave exposure thus seemed to have no effect on ODC activity.

Examination of the time course for 60-Hz amplitude modulated microwave exposure, however, showed a very significant, but transient enhancement of ODC activity in exposed cultures. After 6-hour exposure ODC activity had risen to 1.69 ± 0.18 (± SEM) that of control values, and by 8-hour peaked at 1.92 (± 0.07) times control values. Continued exposure did not maintain ODC activity at an elevated level. After a 12-hour exposure ODC activity had declined

to 1.66 ± 0.12 of control, and after 24 hours of exposure, the ODC activity was at, or slightly below, control values (0.89 ± 0.07).

Since the results presented by Byus *et al* (1987) had shown a maximum ODC enhancement with 16-Hz amplitude modulation of a 450-MHz microwave signal, we decided to test additional AM frequencies for ODC enhancement at the 8-hour exposure time that gave peak enhancement for the L929 cells at 60-Hz AM. The SAR for all experiments was maintained at 3 mW/g. Modulating frequencies of 6, 16 and 600 Hz were tested. Results showed no significant increase in ODC activity for either the 6 or 16 Hz frequencies. Results from the 600-Hz modulation may have produced a slight enhancement of ODC activity, but scatter in these data (1.15 ± 0.25 control values) indicate that if enhancement occurred, it was probably small, and not consistent from experiment to experiment. In addition to the 8-hour exposures, exposure time courses of 2, 4, 6 and 8 hours were conducted for the 16-Hz AM signal. Results, as shown in Figure 2.2, demonstrated no significant enhancement of ODC activity over control values for any of these exposure times.

2.2.2.2. CELL PROLIFERATION AND VIABILITY AFTER MICROWAVE EXPOSURE

Since ODC activity is necessary for replication of DNA and cell proliferation, we subjected cultures of L929 or HL-60 cells to microwave exposures for 72-hour periods. Exposed and control cultures were started with identical inocula, allowed to grow for 72 h in exposure or control conditions, and then assayed for cell viability, doubling time and cell number. Except for the extended time course, exposure conditions were the same as those employed for the work detailed above. As is shown in Table 2.1, neither continuous wave nor 60-Hz amplitude modulation of the microwave signal produced any major alteration in cell growth over the course of the 72-hour time frame.

2.2.2.3. CELL RESPONSE AND COHERENCE OF THE MICROWAVE SIGNAL

A major question surrounding biological response to electromagnetic fields concerns the small amplitude of the fields relative to the random thermal noise to which the cell is exposed. Weaver and Astumian (1990) have hypothesized that one of the ways in which cells may be able to detect such weak electromagnetic fields is through a process of signal averaging over time. To examine one aspect of such questions, we asked whether during exposure the EMF must maintain coherence for some minimum time interval in order to produce a biological response.

To perform these experiments the electrical engineering group wrote a computer program which controlled a function generator (details are provided in Chapter 3 of this report) to provide a frequency switching of the amplitude modulation signal. Amplitude modulation was alternated from 55 Hz to 65 Hz at intervals chosen by the researcher. To assure incoherence in the frequency switching, a random time element, from 0 to 50 ms, was subtracted from each coherence interval. For a coherence time of 0.1 s, amplitude modulation would be switched from 55 to 65 Hz each 0.1 s (minus the randomly chosen time).

We had previously determined that ODC enhancement occurred after an 8-hour exposure if the microwaves were amplitude modulated at 60 Hz, but not if modulation was at 16 Hz. To

assure that 55 and 65 Hz modulation would produce the same enhancement as 60 Hz, we performed 8-hour exposures with the microwave signal amplitude modulated at each of these frequencies. The results, shown in Figure 2.3, demonstrate that either modulation frequency, used alone, gave ODC enhancement equivalent to that obtained with 60 Hz.

A series of exposures was initiated, all using the 8-hour interval shown to produce a maximal ODC enhancement for the 60-Hz AM signal. Coherence times were 0.1, 1.0, 5.0, 10.0 and 50.0 s. Average ODC enhancements produced by each of these times are displayed in Figure 2.4. No enhancement of ODC activity was produced when coherence times of 0.1 or 1.0 s were employed. Coherence times of 10.0 and 50.0 s, however, produced ODC enhancement equivalent to that obtained by amplitude modulation at 60 Hz. A 5.0 s coherence time yielded an ODC enhancement (1.54 ± 0.21 control values) that was an intermediate value between control and full AM microwave enhancement.

These results indicate that, regardless of the question of field strength, cells must perceive a coherent signal for some minimum time interval in order to respond fully. In this case the interval is between 1.0 and 10.0 s.

2.2.2.4. ELF EXPOSURE

Enhancement of ODC activity was minimal unless the microwave signal was amplitude modulated at approximately 60 Hz. We interpreted such enhancement as an ELF effect, stemming from the cell's demodulating (by some unspecified mechanism) the modulated microwave signal. If this were true, then exposure of the L929 cultures to a 60-Hz, sinusoidally varying electromagnetic field should also be capable of producing an equivalent enhancement of ODC activity. To test these results a series of exposures was made using a Helmholz coil system which produced a horizontal magnetic field.

L929 cultures exposed to 60-Hz ELF magnetic fields of 100 μT for 4 or 8 hours showed no enhancement of ODC activity over control cultures. Decreasing the magnetic field intensity to 10 μT produced no enhancement after 8 hours of exposure, but a 4-hour exposure resulted in a two-fold enhancement (2.04 ± 0.21). This is equivalent to that obtained in the AM microwave exposures after 8 hours (Figure 2.5). An extremely low frequency signal, whether a simple sinusoidal EMF or used for microwave modulation, was thus shown to produce enhancement of ODC activity.

ELF exposures were also attempted using HL-60 cells. These experiments were done in a solenoid exposure system which allowed two cell populations, in concentric chambers, to be simultaneously exposed to the same magnetic field, but different electric fields. These studies were conducted prior to the L929 work, and so the time course was only taken to 6 hours. Results (Table 2.2 and Figure 2.6) showed no significant differences in ODC activity for either of the ELF exposed populations as compared to control cultures. The general rise in ODC activity seen over the first 120 min of the experiment stemmed from stimulation of the cells by fresh, serum-containing medium immediately before onset of exposure. In order to avoid the ODC activity rise resulting from serum stimulation, the experiments utilizing L929 cultures were

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all done with cultures which had been set up the day before, and which did not experience a change of culture medium prior to onset of exposure.

2.2.2.5. CELL RESPONSE AND COHERENCE OF THE ELF SIGNAL

Cell response relative to coherence time was investigated for the 60-Hz ELF system. Since a coherence time of 1.0 s had produced no enhancement, and a 10.0 s coherence time full enhancement in the microwave system, these times were initially selected for evaluation. All exposure times were for 4 h. Data are presented in Figure 2.5. A coherence time of 1.0 s produced no enhancement of ODC activity over controls, but a 10.0 s time gave an enhancement equivalent to that seen with the unaltered 60-Hz signal. These results follow the same pattern as those obtained for the AM microwave coherence series. At present the full series of coherence times have not been completed, but are in progress. Preliminary results indicate that the series will follow the same pattern as that obtained for the microwave exposures, with 5.0 s coherence time yielding an intermediate value for ODC enhancement.

2.2.2.6. cDNA PROBE FOR THE ODC mRNA

The response of L929 cultures to either amplitude modulated microwave or ELF exposure conditions has shown that specific activity of the ODC enzyme will approximately double. It cannot be determined from these data whether the doubling comes about due to increased transcription of the ODC mRNA, increased translation of the ODC mRNA, decreases in the degradation rate of either the ODC mRNA or protein, or some change in the activation state of the enzyme. From what is known of ODC response in other systems (Heby and Perrson, 1990), any one, or combination of, these effects might prove to be the source of the enhancement we observed. Efforts to answer this question have been begun.

We have obtained a pBR322 plasmid (pOD48) from Dr. Coffino of the University of California at San Francisco. This plasmid contains a cDNA probe for the murine ODC gene (McConlogue *et al.*, 1984). The probe will be used, in dot blotting and Northern blotting techniques, to assess the levels of ODC mRNA after different exposure conditions. To assure adequate supplies of the ODC probe, we have transfected the *E. coli* HB101 strain with the plasmid and have obtained transfected clones as determined by a tetracycline resistance marker. These clones will be further assayed to assure positive transfection, and then large preparations of plasmid will be produced for use in experiments. Restriction maps of the original plasmid and plasmids obtained from transfected cells have been shown to be identical.

2.3. ALTERATION OF rRNA AND mRNA TRANSCRIPTION AND PROCESSING FROM EXPOSURE TO ELF FIELDS

One of the more intriguing biological responses reported as the result of exposing cells to an electromagnetic field is the transiently enhanced level of various mRNAs reported by Goodman, Henderson and their colleagues (Goodman & Henderson, 1987, 1988; Goodman *et al*, 1983, 1989). HL-60 cells exposed to low intensity, horizontal magnetic fields from a Helmholz coil system experienced as much as 3-4 fold increases in total amounts of mRNAs for c-myc,

β-actin, histone H2B, and fos, within 40 minutes of the onset of exposure. Such enhancement was not found for globin mRNA, but the globin genes are not expressed in the HL-60 cell line. The peak in mRNA levels apparently occurs at an earlier timepoint if the intensity of the magnetic field is elevated.

These results are intriguing, but difficult to interpret. Elevation of proto-oncogene mRNA levels is of clear interest in light of the possible association of electromagnetic field exposure with increased incidence of leukemia and other cancers. Enhancement, however, seems to occur for virtually any of the mRNAs tested, provided they are normally expressed in the HL-60 cell line. Other researchers have not replicated this effect. Byus et al. (1987, 1988), who have demonstrated increased specific ODC activity in cultured cells exposed to amplitude-modulated microwave or ELF fields, have detected no change in ODC mRNA levels as assayed by dot or Northern blot techniques (Byus et al., 1990). We have made an extensive series of exposures to assess effects of ELF fields on steady state levels of specific mRNAs. These results are reported below.

Our other transcription studies have shown that exposure of HL-60 human leukemia cells to 60-Hz ELF did produce a transient enhancement in transcription, as measured by pulse incorporation of ^{3}H-uridine into total cellular RNA. These studies, involving irradiation of cells in concentrically partitioned dishes within a solenoid exposure chamber, demonstrated that the enhanced incorporation of uridine could be attributed to the magnitude of the induced electric field. During this past year, work has continued on this field-induced enhancement of transcription. Efforts have been made to determine (1) whether enhancement affects many genes, or is limited to a small number, and (2) whether ELF fields affect the processing of the newly transcribed RNA.

Results of the transcription studies, as detailed below, indicate that transcription and processing of the ribosomal 45S precursor are selectively affected by ELF field exposure.

2.3.1. METHODS

2.3.1.1. ELF Exposures

ELF exposures were conducted using either solenoid or Helmholz coil systems. The magnetic field of the solenoid system was perpendicular to the growth surface of the cultures, and homogeneous, but the induced electric field increased radially from the center of the dish. In the Helmholz system the coils were positioned vertically, providing a horizontal magnetic field (parallel to the growth surface) and, thus, a relatively uniform electric field across the growth surface. HL-60 cells (5×10^6 cells/ml) to be exposed in the solenoid system were placed in the innermost and outermost annular rings of a multi-chamber culture dish. This provided cultures exposed to the same magnetic field, but different electric fields. Cells exposed in the Helmholz system were grown in standard 25 cm^2 flasks. Exposure times for either system were 0, 10, 20 and 40 minutes. Magnetic fields of 10, 100 1,000 and 10,000 μT sinusoidally varying at 60 Hz were used.

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2.3.1.2. RNA Extraction and Processing for Blotting

RNA Isolation for dot blotting and Northern blotting techniques was done by the modified acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). 5.0 to 20 x 10^6 HL-60 cells were lysed in 800 µl of denaturing solution (4.0 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7; 0.5% sarcosyl; 0.1 M β-mercaptoethanol, added fresh) and vortexed rapidly for 15 seconds. 80 µl 2.0 M sodium acetate (pH 4) were added, and the sample vortexed an additional 15 sec. To this, 800 µl phenol (Tris, pH 7.4, saturated) and 160 µl chloroform (with iso-amyl alcohol in 24:1 ratio) were added, and vortexing was done for 30 s. The preparation was placed into an ice bath for 15 min, then centrifuged at 10,000 x g for 20 min at 4°C.

Most (~ 800 µl) of the aqueous phase was transferred to a clean tube without disturbing the organic phase or the interface. 800 µl isopropyl alcohol was added, the sample vortexed briefly, placed at -70°C for 30 min, and then centrifuged at 10,000 x g for 20 min at 4°C. The pellet was dissolved in 300 µl denaturing solution, 650 µl absolute ethanol was added, and the preparation placed at -70°C for an additional 30 min. Centrifugation was repeated, the supernatant discarded, and the pellet washed with 1.0 ml 75% ethanol. Following an additional centrifugation the RNA pellet was resuspended in 30 µl Tris-EDTA buffer (TE: 10 mM Tris, pH 7.5 and 0.1 mM EDTA), and RNA concentration determined by UV analysis.

2.3.1.3 Analysis of mRNA by Dot or Northern blotting

Dot blots. Dilutions of each RNA sample were made and loaded to the nylon membrane in 200 µl 10X SSC via vacuum filtration. The membrane was washed 2X with 200 µl 10X SSC.

Northern blots. 15 µg of RNA from each sample was glyoxal/DMSO denatured and loaded to a 1.2% agarose gel. The gel was electrophoresed for 4 h at 50 V and the RNA was then transferred to a nylon membrane via capillary action with 10X SSC overnight. RNA was fixed to membranes by baking. Prehybridization of membranes was for 3 h at 44° C in 25 ml prehybridization buffer (50% formamide; 5X SSPE; 5X Denhardt's solution; 0.1% SDS; 150 µg/ml denatured salmon sperm DNA). Hybridization was accomplished by adding 1.0 ml denatured ^{32}P-labeled cDNA for c-myc, beta-actin, or histone H2B and incubating at 44° C for 18 h. After hybridization the membrane was washed sequentially 2X in 250 ml of 6X SSPE, 0.1% SDS at room temperature, 15 min; 2X in 250 ml 1X SSPE, 0.1% SDS at 44° C, 15 min; and finally, in 250 ml 0.1X SSPE, 0.1% SDS at 50° C, 20 min. The moist membrane was wrapped in plastic film placed into a cassette with X-ray film and exposed at -70° C for 24 h. The film was developed with GBX developer and fixer. Densitometry was done using a BioImage Visage 60 computer analysis system to determine optical density, or integrated optical density for each spot or band.

2.3.1.4 RNA Preparation for S_1 Transcription Assay

HL60 cells, positioned in the outer compartment of organ culture dishes, were irradiated for 90 minutes in a solenoid device (60 Hz, 1 mT magnetic field). For the last 15 minutes of irradiation, the cultures were pulse labeled with ^{3}H-uridine (20 µC/µl). Cells were collected in

50 ml conical tubes and placed on ice. The organ tissue culture dishes were then washed with 3 ml of cold 1 X PBS. The HL60 cells were spun straight away at 2000 rpm for 5 minutes, the supernatant removed and the resulting pellet suspended in 1.0 ml of cold 1 X PBS. The pellet was tapped to dislodge the cells and the suspension was transferred to a 2.0 ml microtube which was spun at 3000 rpm for 5 min. The supernatant was removed and the pellet suspended in 0.9 ml of cold NP-40 lysis buffer. To ensure complete lysis the tube was inverted several times and the sample was left on ice for 2 minutes. After this time the sample was centrifuged at high speed (setting #10 for microfuge) for 5 min to pellet nuclei. The pellet was suspended in 0.9 ml of SDS buffer. Just before use stock DNase was diluted with 1 X SSC. 100 µl of DNase were added per sample and this was incubated at 37° C for 15 minutes. After the incubation period an equal volume (1.0 ml) of a 1:1 phenol/chloroform:isoamyl alcohol mixture was added. The sample was then inverted several times and centrifuged at high speed for 2 minutes. The aqueous phase was removed and to this was added 1.0 M NaOH so that the final concentration with respect to NaOH was 0.2 M. The sample was left on ice for 10 minutes and then neutralized with an equivalent volume of 1 M HCl. A 1/10-volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol were then added to the sample, which was then placed on ice for 30 minutes to concentrate the RNA. The sample was centrifuged at high speed for 5 minutes and the RNA pellet washed with 70% ethanol. The residual ethanol was removed from the pellet, but the pellet was not allowed to dry completely since this made resuspension in the hybridization buffer very difficult. The sample was then ready to be analyzed in the S1 transcription assay.

2.3.1.5. S_1 TRANSCRIPTION ASSAY

Each sample of pulse-labeled RNA was suspended in a 20-µl volume of S_1 hybridization buffer (40% formamide, 40 mM PIPES pH 6.4, 400 mM NaCl, 1 mM EDTA). A 1X volume of cold DNA probe (denatured, if the probe was double-stranded) was added, and samples heated to 65°C to stretch out the RNA sample. Hybridization was carried out overnight at 30°C. A control sample without probe was treated the same way. The next morning 300 µl of a digestion mix were added to each sample (150 µl 2X S1 nuclease buffer, 147 µl sterile water, 250 U S_1 nuclease, 3 µl denatured salmon sperm DNA [2 µg/µl], 2X S_1 Nuclease Buffer [0.56 M NaCl, 0.1 M sodium acetate pH 4.5, 9 mM $ZnSO_4$]). Samples were then incubated for 60 minutes at 30° C. After this incubation, the reaction was stopped by the addition of 80 µl of stop buffer (4 M ammonium acetate, 20 mM EDTA pH 8.0, 40 µg/ml tRNA) followed by 1.0 ml 95% ethanol, and samples were left on ice for 10 min. Precipitates were collected on glass filters using a filter bank. Filters, containing the labelled RNA samples hybridized against their respective probes, were mixed with 4.0 ml scintillation cocktail and counted in a scintillation counter. Transcription is related to the difference in the counts with and without the probe.

2.3.1.6 PULSE-CHASE LABELING AND AGAROSE GEL ELECTROPHORESIS

HL60 cells were irradiated in the solenoid chamber in outer compartments of organ culture dishes with a 60-Hz 1-mT magnetic field for various times. During the last 15 minutes of irradiation, the cultures were pulse-labeled with ^{3}H-uridine at 20 µCi/ml. At the conclusion of the labeling period, the medium was removed from the cultures, the cultures were washed with PBS, and fresh culture medium containing 100X excess of cold uridine was added. After various

chase periods, the total cellular RNA was isolated by a modified one-step procedure employing a guanidinium isothiocynate lysis buffer. The precipitated RNA was dissolved in 20 µl of sterile water, and 20 µg of the RNA was denatured by incubation in denaturation buffer (0.5X MOPS, 6.5% formaldehyde, 50% formamide) at 55°C for 15 minutes. Following incubation, 2 µl of 10X loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) were added and the mixture loaded into individual wells of a 1.2% agarose/2% formaldehyde gel. The gel was electrophoresed in 1X MOPS buffer at 5-6 V/cm until the dye front traveled 2/3 the length of the gel. After the gel was stained with ethidium bromide and photographed, the lanes of interest from the gel were cut out and each sliced into 20 equal pieces using a razor blade device. Each piece sliced from a gel was placed into a separate scintillation vial, 1 ml of water was added to each, and the gel pieces melted in a microwave oven. After melting, scintillation fluid was added and the samples counted in a scintillation counter.

2.3.2. RESULTS

2.3.2.1. STEADY STATE LEVELS OF C-MYC, β-ACTIN, AND HISTONE H2B mRNAS

Analysis of steady state levels of c-myc, β-actin and histone H2B mRNAs, whether by densitometry of autoradiographic results from Northern blotting, or from autoradiograph densitometry or actual counts from dot blots, gave a consistent result for all conditions of exposure examined. In each case there were no significant deviations in quantities of the mRNAs for any of the three genes. Representative results from exposure of cells in the Helmholz system and the solenoid system are provided in Figures 2.7 and 2.8. None of the differences in values among the exposed and control specimens for any time point were significant relative to the normal level of variation observed among replicate samples.

Initially, we had felt that in the dot blot assay non-specific binding of the cDNA probes to RNAs other than their specific message might be obscuring any gene-specific changes. Northern blot analyses, however, showed very clean and specific bindings of the probes to appropriately sized mRNAs (c-myc at 2.4, β-actin at 2.0 and histone H2B at 0.8 kb), and densitometric analysis of these bands also showed no significant differences between exposed and control samples (Figure 2.8).

Since we could not replicate the reported results of the Goodman and Henderson group, it was arranged that one of our group, Dr. David Krause, would visit the Goodman-Henderson laboratory. While he was there HL-60 cultures were exposed under the ELF conditions they report have consistently yielded mRNA enhancements in their hands. The exposed and control cells were aliquoted into 2 groups each, with Dr. Krause extracting RNA from one set of samples, and the postdoctoral associate from the Goodman-Henderson group processing the other set. Dr. Krause brought one-half of the RNAs to Catholic University for dot and Northern blottings. Although dot blot analysis showed considerable enhancement for specific mRNAs, Northern blot analysis showed that binding of the cDNA probes to the blots had not been to the desired mRNAs. Indeed, no changes were observed in the levels of mRNA for c-myc or β-actin (the position of the histone H2B mRNA was obscured), but intense binding was observed at a position corresponding to an RNA of 0.8 kilobases. The Goodman-Henderson group did not

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find this intense band in their samples, but did report some enhancement of the three mRNAs examined. The discrepancy between these two sets of results is not clear, but it appears that the ELF-exposed RNA samples brought to Catholic University were contaminated by cDNA probe, which would band at the position observed on Northern blots, and would account for the intense enhancement observed in dot blots. Since we observed no enhancement for the specific mRNAs in question, and since our own exposures have consistently shown no enhancement of steady state levels for these mRNAs, we have abandoned this set of experiments. It is not clear why our results differ consistently from those reported by the Goodman-Henderson group.

2.3.2.2 INCORPORATION OF LABELED URIDINE INTO, AND PROCESSING OF, THE 45S rRNA

Previous work had shown that 60-Hz ELF exposures (magnetic field of 1 mT) in the solenoid system enhanced incorporation of ^{3}H uridine (pulsed during the last 15 min of exposure) into total cellular RNA. We have now completed studies on uridine incorporation using continuous labeling techniques in which the ^{3}H uridine was present for the entire time of exposure. Results (Figure 2.9) surprisingly, showed no differences from continuous labeling between exposed and control cultures.

Since pulse labeling had shown enhanced incorporation of uridine by exposed cultures, but continuous labeling over exposure periods of 30 to 60 min showed no difference, it was hypothesized that exposure to the ELF field might enhance both the rate of synthesis of the RNA, and the rate at which it is processed or degraded within the cell. As a consequence, a series of experiments was conducted to determine if particular species of RNA were enhanced following a short pulse, and to follow their processing during subsequent chase periods. To determine any major species of RNA into which the labeled uridine was incorporated, a 15 min pulse of ^{3}H-uridine was provided at the completion of a 60-min exposure. The extracted RNAs were size fractionated by agarose electrophoresis. Gel lanes were cut into multiple slices, and counts per minute in each slice determined. Results from this work are presented in Figure 2.10.

The only RNAs present in sufficiently high quantities to provide readily definable peaks were sized at 45S, 28S, 18S, and 5S. On the figure, fraction 5 marks the approximate position of the 45S, fraction 9 the 28S, fraction 12 the 18S, and fraction 19 the 5S RNA species. Pulse-chase studies of control populations showed that label initially incorporated into the 45S peak shifted to peaks of 28S and 18S during the chase periods (Figure 2.10). These results established the 45S peak as the 45S rRNA precursor, and the 28S and 18S peaks as ribosomal rRNAs formed from the precursor. The 5S peak would contain a number of RNA species in addition to the 5S rRNA. No individual mRNA species were present in sufficient quantities, in either control or exposed samples, to be detected by these techniques.

Comparison of the profiles from exposed and control cells harvested immediately after the pulse label period showed a 40-80% increase in the incorporation of labeled uridine into the 45S rRNA peak of exposed relative to control cells (Figure 2.10). These data indicate that the major incorporation of 3H-uridine into total cellular RNA, which we had observed previously, may be accounted for by incorporation of the label into rRNAs.

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In order to follow processing of the 45S rRNA species the pulse chase experiment was altered. Tritiated uridine was added for a 15 min pulse at the onset of exposure, and then a chase period, accompanied by addition of a 1000X excess of cold uridine, was begun. Cells were harvested at various times during the chase period, and processed by electrophoresis and size fractionation as before. Counts per minute in the 45S rRNA fraction were analyzed. These pulse-chase experiments showed that the 45S RNA precursor is processed more rapidly during ELF exposure. Figure 2.11 shows the decline in the levels of label incorporated into 45S nuclear RNAs for control and exposed cells. The half-life of the 45S RNA species, presumably the rRNA precursor, is 85 min for the irradiated cells vs 115 min for the control cells. ELF exposure thus decreased the 45S rRNA processing time by slightly more than 25%.

2.3.2.3. SPECIFIC TRANSCRIPTION ASSAYS OF c-MYC, β-ACTIN AND rRNA GENES

To facilitate the assessment of how an ELF field affects the transcription of specific genes, a new specific transcription assay was developed. The established assay, the nuclear run-off (or, nuclear run-on) assay, is very complex, involving the isolation of intact nuclei and *in vitro* transcription in the presence of radiolabeled nucleotides, and isolation of specific transcripts by filter-hybridization. Quantification is by the level of film exposure during autoradiography. This procedure is not only very cumbersome but is also not very reproducible, since it is totally dependent upon the viability of the nuclei. Further, it is not very quantitative, since it depends upon the intensity of autoradiographic exposure. The new, rapid procedure, developed during this year circumvents many of these problems. The procedure provides an estimation of a gene's transcription rate from the amount of ^{3}H-uridine pulse-labeled nuclear RNA that is protected from S_1 nuclease digestion by hybridization of a gene-specific probe to the nuclear RNA. Since S_1 nuclease preferentially digests single-stranded nucleic acids, the radioactivity associated with double stranded, and hence protected, RNA would primarily reflect new transcription from the corresponding gene.

The S_1 nuclease transcription assay has been used to assess the influence of ELF fields on the 45S ribosomal, c-myc, and actin gene transcription rates. To date, a total of 20 transcription rate experiments have been done examining the effects of a 90 minute exposure to 1-mT 60-Hz ELF radiation on transcription of the 45S ribosomal and c-myc genes in human HL-60 cells. Previous experiments have indicated that general transcription is enhanced by this exposure, and, as detailed above, most, to all, of the increased transcription seems to be for the 45S rRNA gene. For samples assayed by the new S_1 nuclease protocol there was an average of 30% enhancement in transcription rate for the 45S rRNA gene. In the one sample for which there was a reduction, the reduction was 6% (changes of 5% or less are not considered significant. For the c-myc gene, less than 5% change was seen in 5 of the 7 experiments, while two of the experiments were uninterpretable due to salt carry-over. These results suggest that the ELF enhancement is selective, capable of affecting some genes but not others. These data correlate with our other work indicating no change in steady state levels for the c-myc mRNA, but increased rates of transcription for the 45S rRNA precursor.

Most recently a β-actin plasmid clone was obtained and the β-actin plasmid purified. This plasmid will be used as a probe for further transcription rate studies. Additionally, the ODC plasmid clone now available (described in Section 2.2) will be utilized for transcription rate

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studies of the ODC gene. These data will help us evaluate the basis for the enhanced ODC activity seen after either AM microwave or ELF magnetic field exposures.

2.4. DISCUSSION

2.4.1. ELECTROMAGNETIC FIELDS AND ENHANCEMENT OF ORNITHINE DECARBOXYLASE ACTIVITY

Ornithine decarboxylase is an interesting target of investigation of electromagnetic field effects for two reasons: (1) The enzyme is readily induced by a number of ligands, and different signal transduction pathways may be utilized by different ligands; and (2) ODC is essential for the production of polyamines, and therefore required for DNA replication and cell proliferation (reviewed by Heby and Persson, 1990; Pegg, 1988). Byus *et al.* (1987, 1988) have shown that ODC activity may be elevated by exposure of cell cultures either to 60 Hz, sinusoidally varying fields, or to 450-GHz microwaves modulated at 16 Hz. Additionally, they have shown alterations in protein kinase activity in cultured cells as the result of modulated microwave exposure (Byus *et al.*, 1984). Since protein kinases are involved with a variety of second messenger systems in signal transduction, these data indicate a possible effect of ELF or modulated microwave signals upon cell surface receptors, altering the activity second messenger systems within the cell.

Our results demonstrate both ELF and modulated microwave alterations of ODC activity in the L929 cell line of murine fibroblasts. As reported by Byus *et al.* (1988) for other cell lines, modulated, but not continuous wave, microwaves did enhance the specific activity of ODC in L929 cells. A number of differences are evident, however, between our data and theirs. We obtained significant effects with 60-Hz AM but not with 16-Hz AM microwaves, while they saw virtually no enhancement with 60-Hz AM, but maximal response at 16 Hz. Perhaps the differences in the carrier frequencies (450 Hz vs. 915 Hz) can explain some of this effect. We judge this unlikely, however, since continuous wave signals produced no major enhancement of ODC activity in either system. An additional difference is seen in the timing of the maximal peak of ODC activity. In both our system and that of the Byus group the elevation of ODC activity was a transient phenomenon. In our case the peak of activity was observed at 8 h from onset of exposure, while they saw peaks occurring at 2 to 5 h, depending upon the cell line used. Differences in exposure regime (they exposed for only 1 h) and differences in cell lines may account for these differences. The major, clear distinction is the difference in effects of different modulating frequencies, and in this case no ready explanation for the differences in results is obvious.

Since amplitude modulation of the microwave signal produced a transient elevation of ODC activity, but a continuous wave signal produced only minimal alteration, we reasoned that it was the extremely low frequency (ELF) of the modulation signal to which cell cultures were responding. Accordingly, a series of experiments was initiated in which sinusoidally varying 60 Hz fields were used to expose cultures. Results produced an elevation of ODC activity, agreeing with the earlier report by Byus *et al.* (1987), comparable to that obtained with modulated microwaves. Timing of the elevation of ODC activity was different than that obtained with AM

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microwaves, however. With a 10-μT, 60-Hz field ODC activity peaked at 4 h. Stronger fields 100 μT) with exposures from 2 to 8 h did not produce this effect. Differences in timing between the ELF and microwave exposures may be due to differences in field conditions which must be determined. None the less, it is clear that a 60-Hz sinusoidally varying signal, with a simple electromagnetic field, or as an amplitude modulating signal for a microwave carrier frequency, is capable of altering ODC activity in a transient manner.

Work by a number of investigators has established the fact that biological parameters may be altered by exposure to electromagnetic fields. Reported effects include elevation of steady state levels of specific mRNAs (Goodman and Henderson, 1987a, 1987b, 1988, 1990; Goodman et al., 1988), enhanced rate of synthesis of the 45S rRNA precursor (Greene et al., 1991), diminished effect of parathyroid hormone on cultured osteoblasts (Luben et al., 1982, 1984), increased Ca^{++} flux across membranes (Walleczek and Liburdy, 1990), increased rates of cell division (Cleary et al., 1990; Cossarizza et al., 1989; Ross, 1990), and alterations in patterns of cell differentiation (Rodemann et al., 1989; Zimmerman et al., 1990). The dilemma in understanding such effects is that many are produced under conditions in which magnetic fields or induced electric fields (in the range of a few mV/cm or even μV/cm) are very small relative to the thermal noise within and around the cell. Weaver and Astumian (1990) have offered the concept of signal averaging over time as a means of overcoming the limit imposed by thermal noise.

Our results from the series of coherence time experiments shows clearly that the ODC response requires the signal, either ELF or AM microwaves, to be coherent for a minimum time interval. Cells were able to respond when frequencies was maintained for 10.0 sec intervals, but showed no response when frequencies were switched at intervals of 1.0 or 0.1 s. A 5.0 s coherence time yielded an enhancement that was intermediate between control and typical experimental values. The concept of signal averaging, thus seems to be a requirement at some level to induce the ODC response. When the coherence time is too small, cells cannot respond, even though exposure is maintained for a full 4 h (ELF) or 8 h (AM microwave). Whether this will account for the cell's ability to respond to a time-varying signal below the thermal noise limit remains to be seen. The concept of signal averaging, however, clearly has some validity.

To summarize, the work on ODC activity has been fruitful in demonstrating a biological response to both ELF and AM microwave fields, and beginning the process of deciphering what conditions of a field determine the biological response.

2.4.2. ALTERATIONS IN RNA SYNTHESIS AND PROCESSING BY ELECTROMAG-NETIC FIELDS

We have conducted an extensive set of experiments designed to determine whether alterations in steady state levels of specific mRNAs, and transcription rates of other RNAs, are altered by exposure to ELF electromagnetic fields. Reports in the literature from Goodman and Henderson and their collaborators (Goodman et al., 1983, 1987, 1989; Goodman and Henderson, 1987, 1989) have documented rapid but transient increases in steady state levels of mRNAs for histone H2B, β-actin, c-myc and other genes which are normally transcribed by the HL-60 cell

line. No rise in hemoglobin mRNA was reported (Goodman and Henderson, 1990), but this gene is not normally transcribed in HL-60 cells. The extent to which these mRNA levels are altered by EMF exposure depends upon the exposure conditions, including frequency and nature of the signal, and amplitude of the magnetic field.

Previous reports have described our experiments to alter mRNA transcription using both solenoid and Helmholz coil exposure systems, and dot and Northern blot detection protocols. The results reported here represent the completion of these efforts. We have been unable, using applied magnetic fields ranging from 1 to 1000 μT, to produce any measurable alteration in steady state levels of mRNAs for the β-actin, c-myc or histone H2B genes. In addition to dot and Northern blotting procedures, we have now used the newly developed S_1 Nuclease Protection Assay to augment these data and show that rates of transcription for the same genes have not varied under conditions which have produced up to 60% increase in rate of transcription for the 45S rRNA precursor (Greene et al., 1991). Efforts to demonstrate ELF field effects on either steady state levels or transcription rates of specific mRNAs is now complete. We cannot duplicate the results of the Goodman and Henderson group, and therefore must conclude that some significant, but not understood, difference exists in protocols, equipment or cell culture systems between the two laboratories. Accordingly, the ability to generate such transient enhancements of specific mRNA levels would seem to require a very tight set of conditions which are not easy to duplicate. Preliminary reports by Phillips et al. (1990) have also indicated that nuclear run-off experiments with HL-60 cells and ELF field exposures have produced no clear enhancement of transcription rates for c-myc and other mRNAs. What this means in terms of environmental exposure and resultant biological effects is not clear, but it suggests that alterations in specific messages are not readily achieved by most exposure conditions.

The work concluded during the past year has painted a clearer picture of the enhancement in overall RNA transcription that was previously reported to result from electric rather than magnetic field strengths (Greene et al., 1991). The original differences were detected with pulse label experiments. Subsequent work, reported here, showed that although transcription rate increases were shown by pulse label, continuous labeling indicated no differences in incorporation of tritiated uridine between exposed and control cultures. The gel/fractionation work and S_1 nuclease protection work detailed in this report offers an explanation of these apparently contradictory data. ELF field exposure does elevate the transcription rate of the 45S rRNA precursor. However, the rate of processing of the 45S precursor into 18S and 28S rRNA molecules also increases in the field. The end result of these effects seems to be that over a period of 30-60 minutes the two rate increases balance, yielding no overall difference in the amount of label retained in the rRNAs between exposed and control cultures. It is, thus, clear that more than one chemical rate in the cells may be altered by ELF exposures, and that the final effect on the cell may be complex and difficult to predict.

2.5. TABLES

Table 2.1. Cell Growth Parameters of L929 Cells after Exposure to Microwaves in a Crawford Cell.

EXPOSURE	% CELL VIABILI-TY	DOUBLING RATE (h)	CELL NUMBER X 10^6
CONTINUOUS WAVE MICROWAVES*			
Control #1	98.5 ± 0.7	30.8 ± 2.0	1.61 ± 0.09
Exposed #1	98.6 ± 0.4	31.8 ± 1.0	1.52 ± 0.08
Control #2	98.4 ± 0.5	25.3 ± 0.6	1.83 ± 0.09
Exposed #2	98.3 ± 0.3	26.0 ± 1.2	1.75 ± 0.14
AMPLITUDE MODULATED MICROWAVES†			
Control #1	94.6 ± 0.5	25.0 ± 0.7	1.84 ± 0.10
Exposed #1	94.9 ± 1.8	26.4 ± 0.5	1.65 ± 0.05
Control #2	98.5 ± 0.5	29.3 ± 1.5	2.10 ± 0.18
Exposed #2	98.5 ± 0.6	29.9 ± 1.0	2.02 ± 0.11

* All microwave (MW) exposures were at 915 MHz at an SAR = 3.0 mW/g.

† The amplitude modulation was 60% at 60 Hz, SAR was 3.0 mW/g.

Table 2.2.

EXPOSURE TIME (minutes)	CONTROL ACTIVITY ODC UNITS	ELF EXPOSED (10 μT, 0.039 mV/m) ODC UNITS	ELF EXPOSED (10 μT, 0.39 mV/m) ODC UNITS
0	2.9	2.9	2.9
15	2.8	3.4	2.8
30	3.9	4.5	5.8
60	6.5	6.4	5.7
90	13.2	11.5	11.6
120	18.8	16.9	18.2
360	3.6	3.4	4.0

2.6. FIGURES

ALTERATION OF ODC ACTIVITY
BY MICROWAVE EXPOSURE
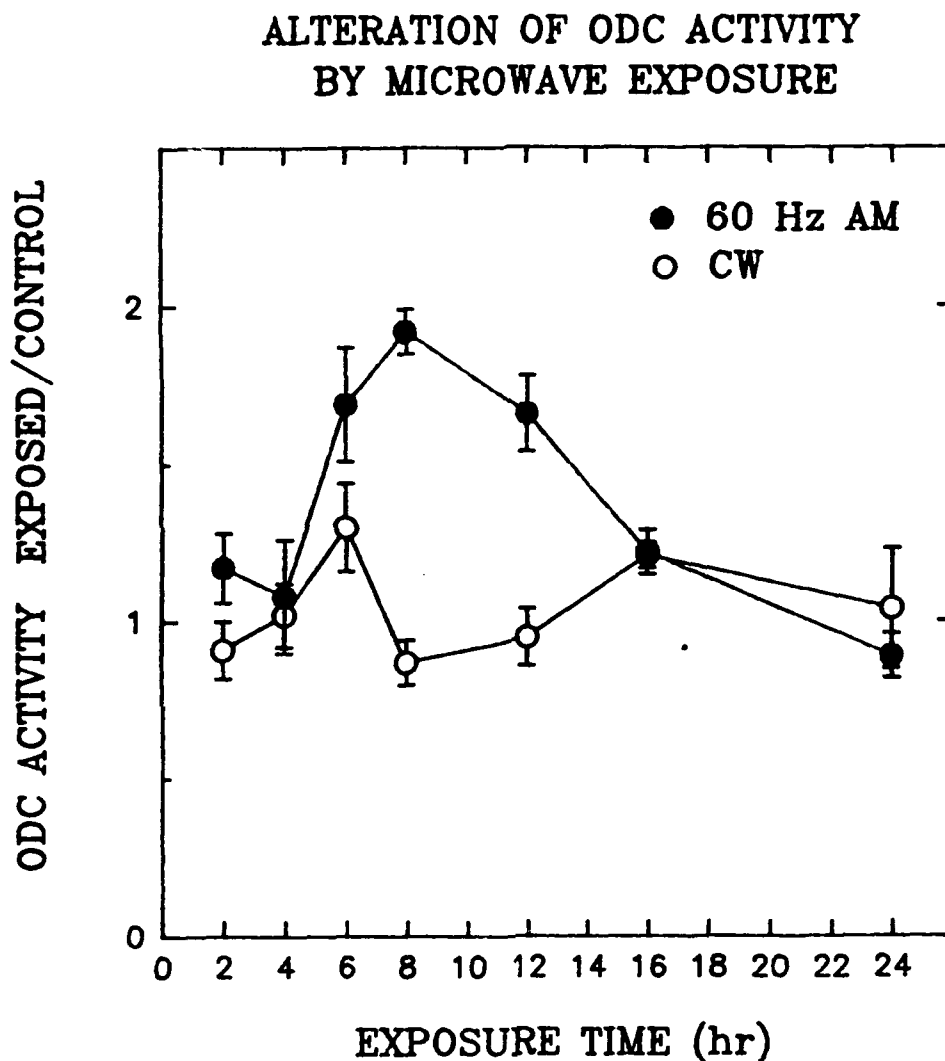
Fig. 2.1. Variations in ornithine decarboxylase (ODC) specific activity of microwave exposed L929 cells for different exposure times. Continuous wave (open circles) and 60 Hz AM (filled circles) microwave signals at an SAR of 3 mW/g were used for different exposure times. Each point represents the mean ± SEM of at least 5 separate experiments. Values are expressed as a ratio of the specific activity of the exposed cultures to that of the controls run at the same time.

ALTERATION OF ODC ACTIVITY
BY MICROWAVE EXPOSURE
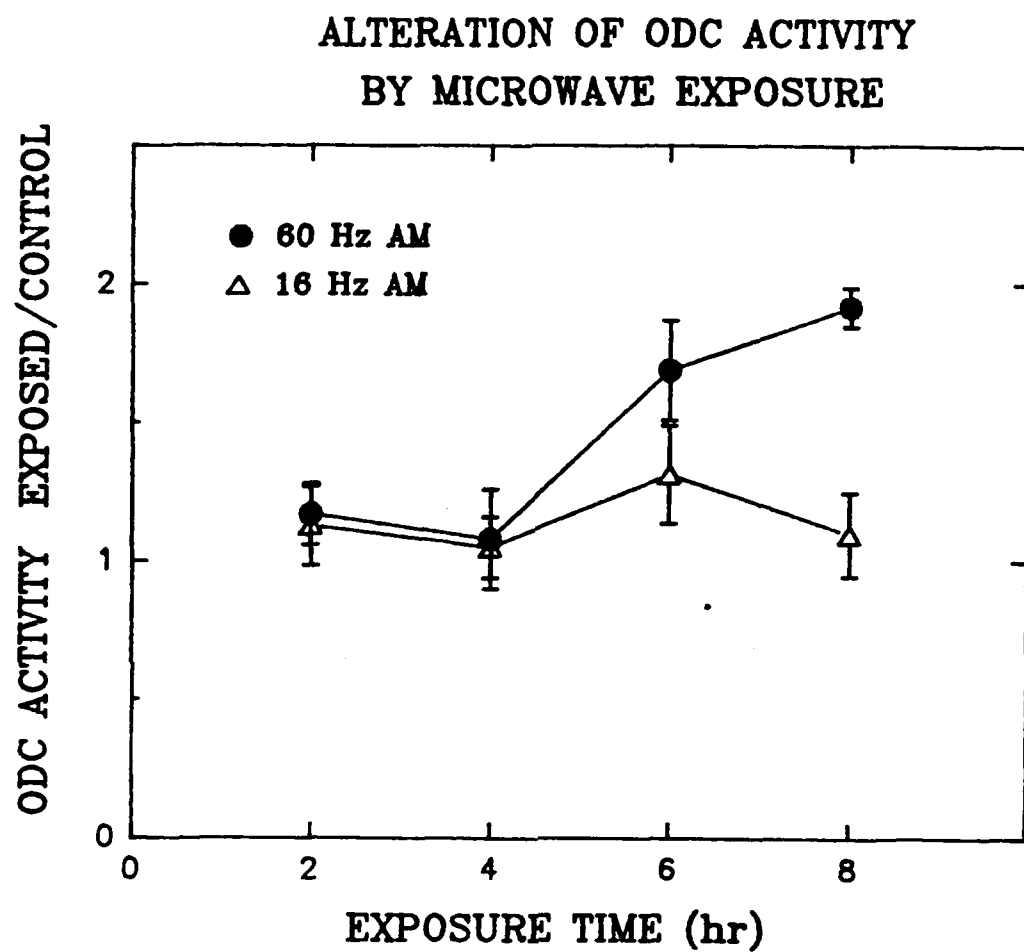
Fig. 2.2. Same conditions as for Figure 2.1, but comparing ODC activities for two different amplitude modulations: filled circles show activities for cultures exposed to 60 Hz AM, and open triangles for 16 Hz AM, microwave signals (hatched bars).

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ALTERATION OF ODC ACTIVITY BY MICROWAVE EXPOSURE
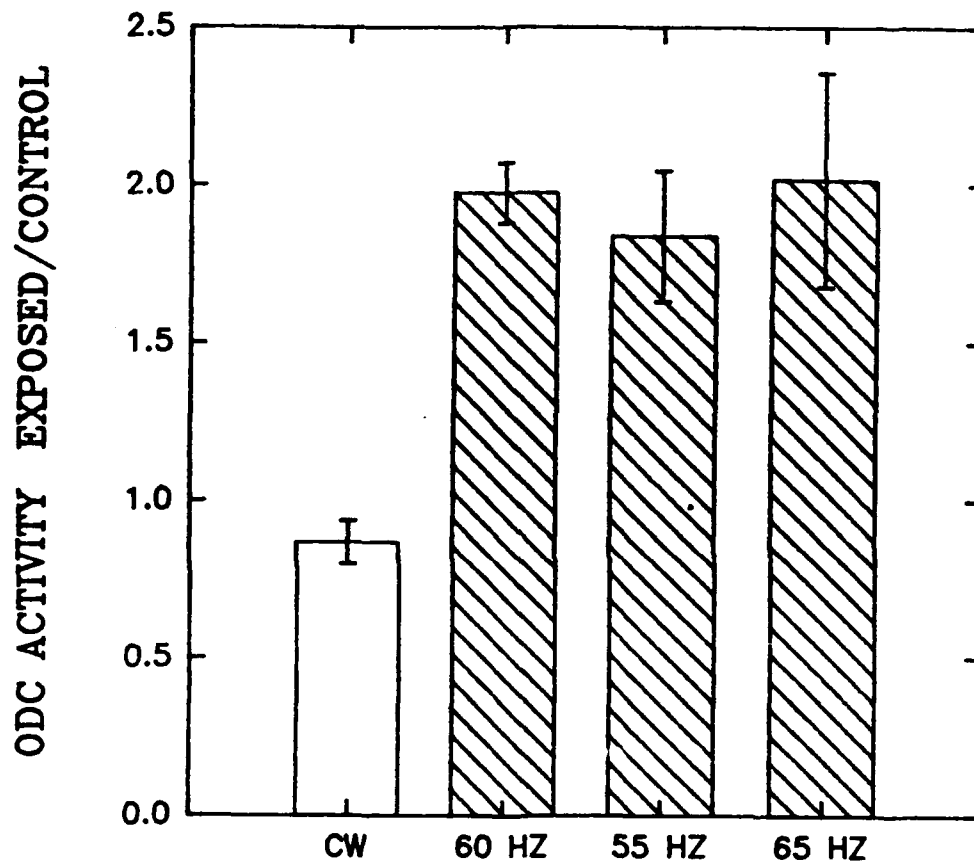
Fig. 2.3. Exposed/control ODC activity ratios comparing results obtained with the continuous wave microwave signal (open bar) and amplitude modulated signals with modulation frequencies of 55, 60 and 65 Hz.

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EFFECTS OF MICROWAVES ON ODC ACTIVITY
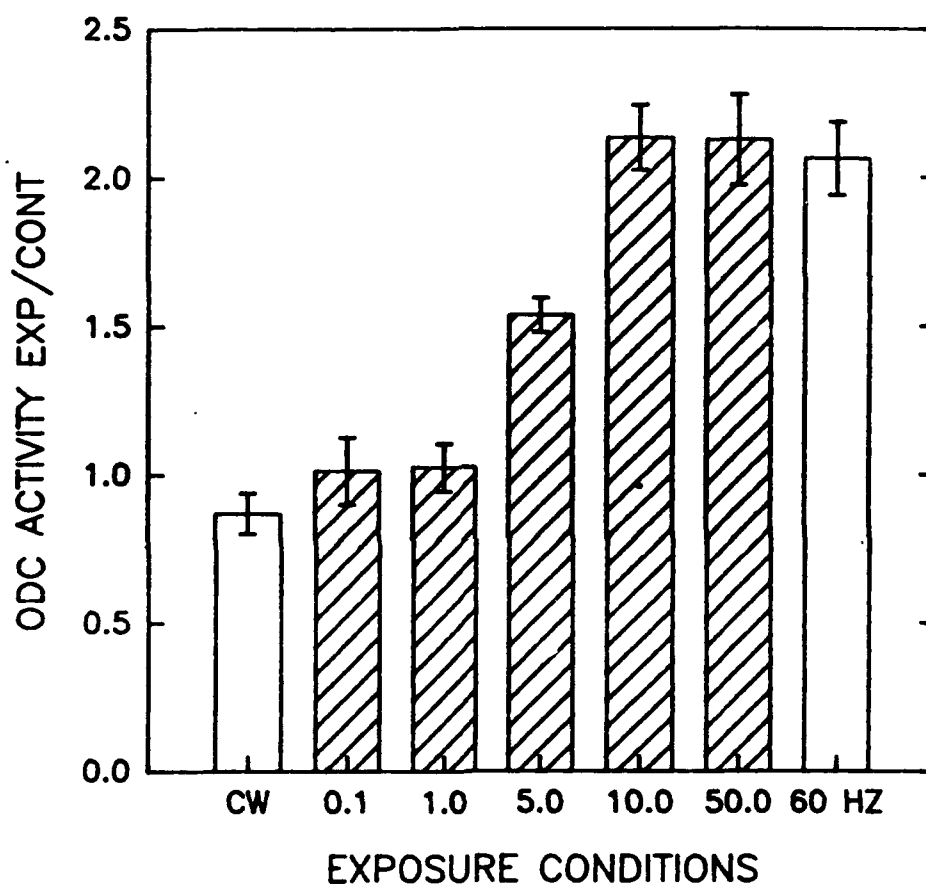
Fig. 2.4. The effect of coherence time interval on ODC enhancement by microwave exposure. Exposed/control ODC activity ratios are given for continuous wave and 60 Hz AM microwave signals (open bars) and for the microwave signal with AM frequency switching from 55 to 65 Hz at specified coherence time intervals (hatched bars). Results for coherence times ranging from 0.1 to 50.0 sec are displayed.

ALTERATION OF ODC ACTIVITY
BY ELF EXPOSURE
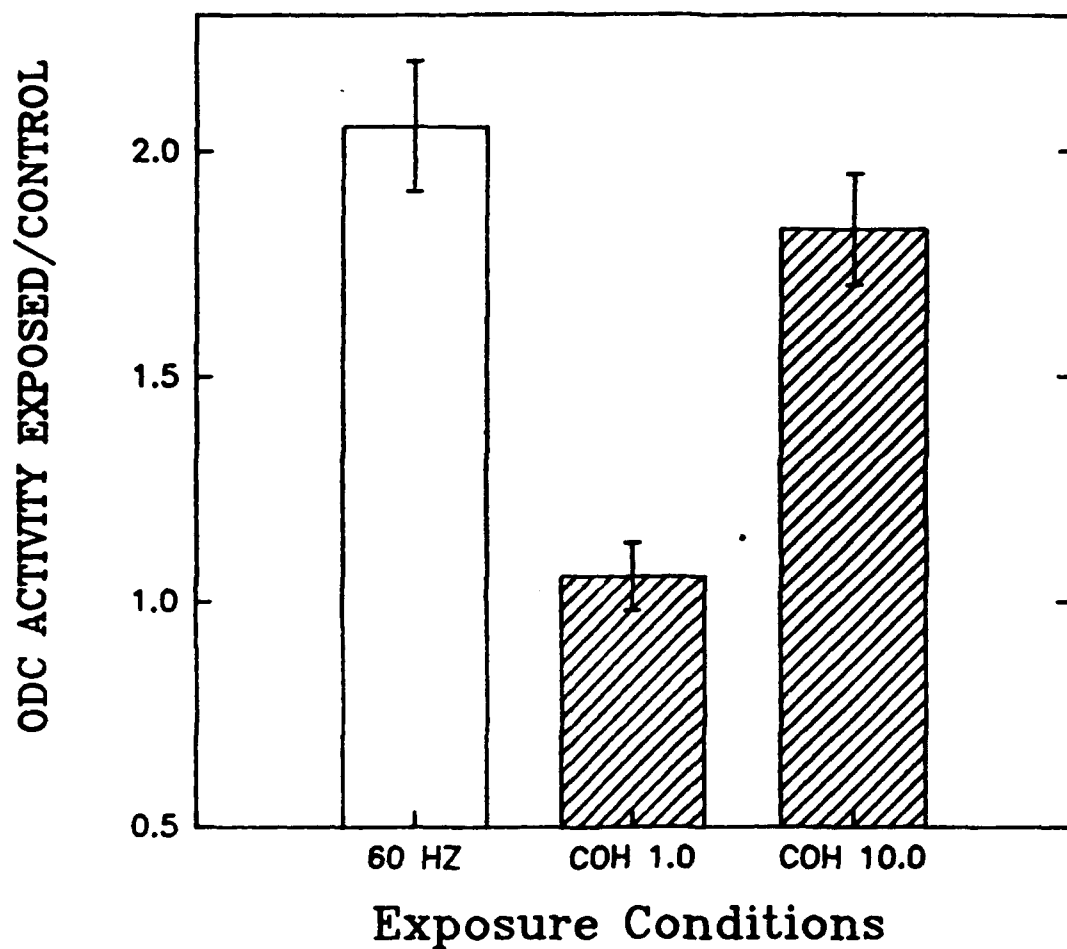
Fig. 2.5. ELF field enhancement of ODC activity is shown for a 60-Hz, sinusoidally varying field (open bar). Also shown are preliminary data on the effect of coherence time variation using ELF field conditions (hatched bars). Results from coherence times of 1.0 and 10.0 s are shown. The effects are remarkably similar to those seen for microwave signals. All exposures were for 4 h with an applied magnetic field of 10 μT.

44
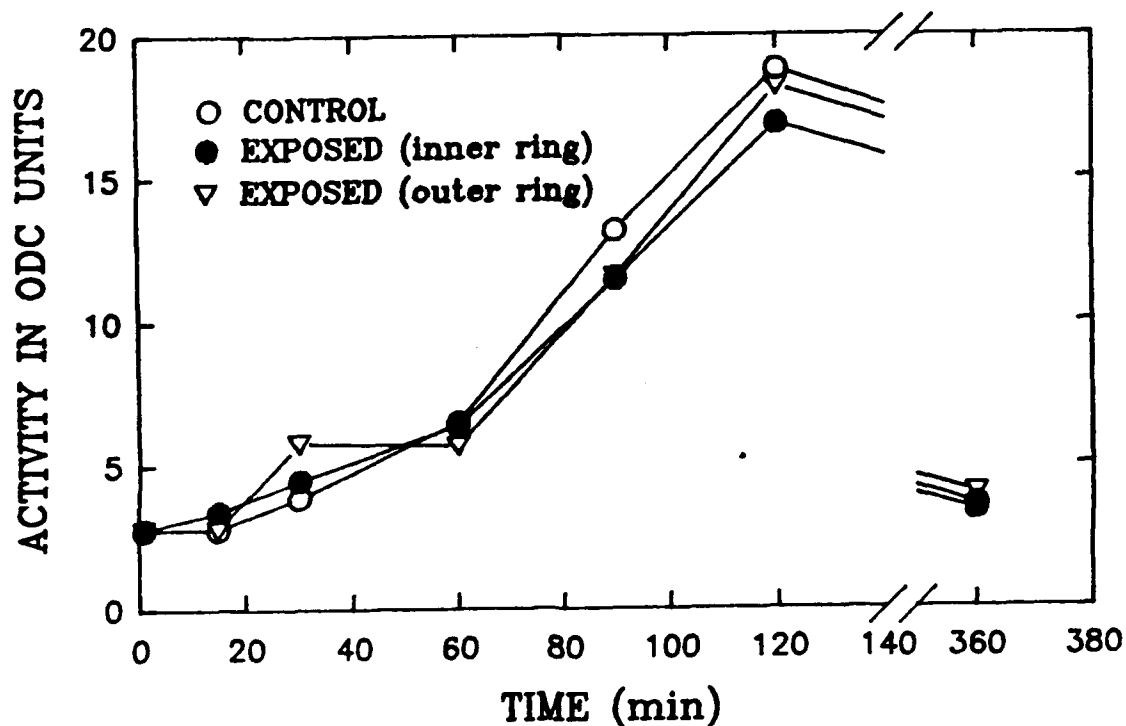
Fig. 2.6. ODC specific activities of HL-60 cells under control (open circles) and ELF field-exposed (filled circles and open triangles) conditions. Culture medium was replaced on the cells at the onset of exposure, resulting in a serum stimulation of ODC activity. Exposure was in a solenoid device using a culture dish with concentric chambers so that two cell populations could be simultaneously exposed to the same magnetic field, but different electric fields (filled circles = inner chamber; open triangles = outer chamber). ELF exposure (applied magnetic field of 1 mT) produced no significant differences in ODC activities from controls.

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HISTONE H2B mRNA LEVELS
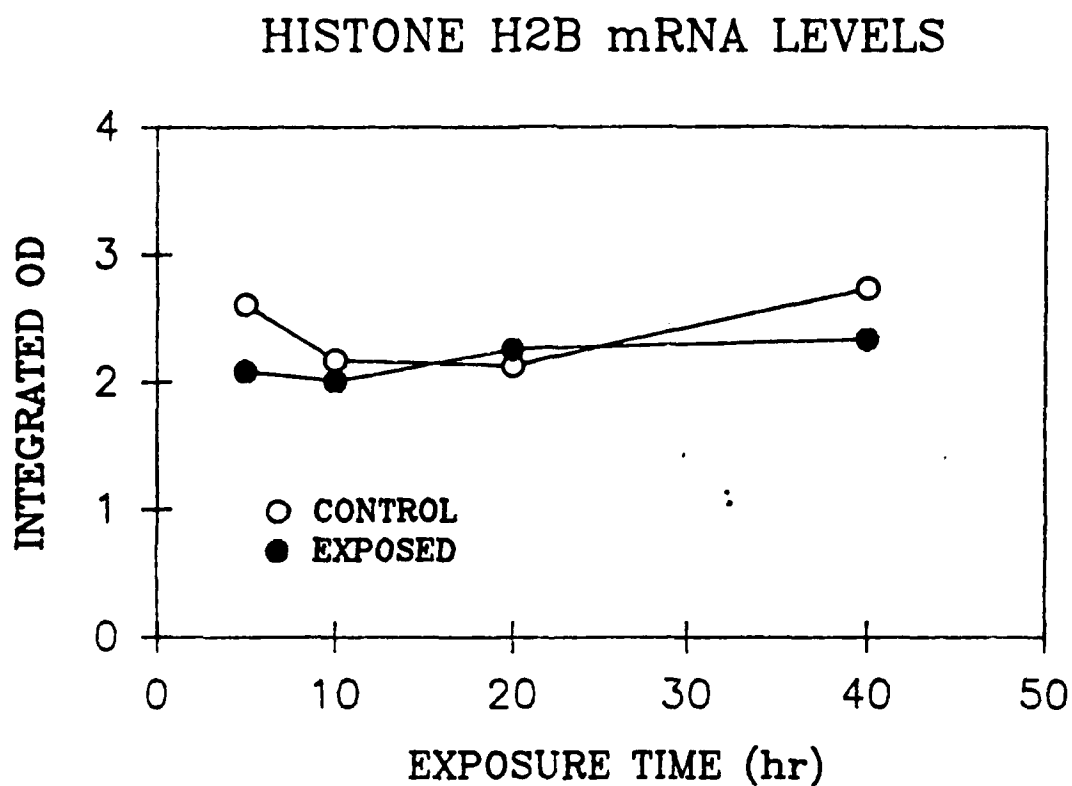
Fig. 2.7 Relative steady state levels of histone H2B mRNA as measured by densitometry. RNA samples were dot blotted and probed for the H2B mRNA. Densitometry was done from an autograph produced from the blotted samples. No significant variation in H2B mRNA level was produced by ELF exposure (10 μT) using a Helmholz coil system.

c-MYC mRNA LEVELS

INTEGRATED OD

3.0

2.5 — ○ CONTROL
● EXPOSED (inner ring)
2.0 — ▽ EXPOSED (outer ring)

1.5

1.0

0.5

0.0

0   10   20   3C   40

TIME (min)
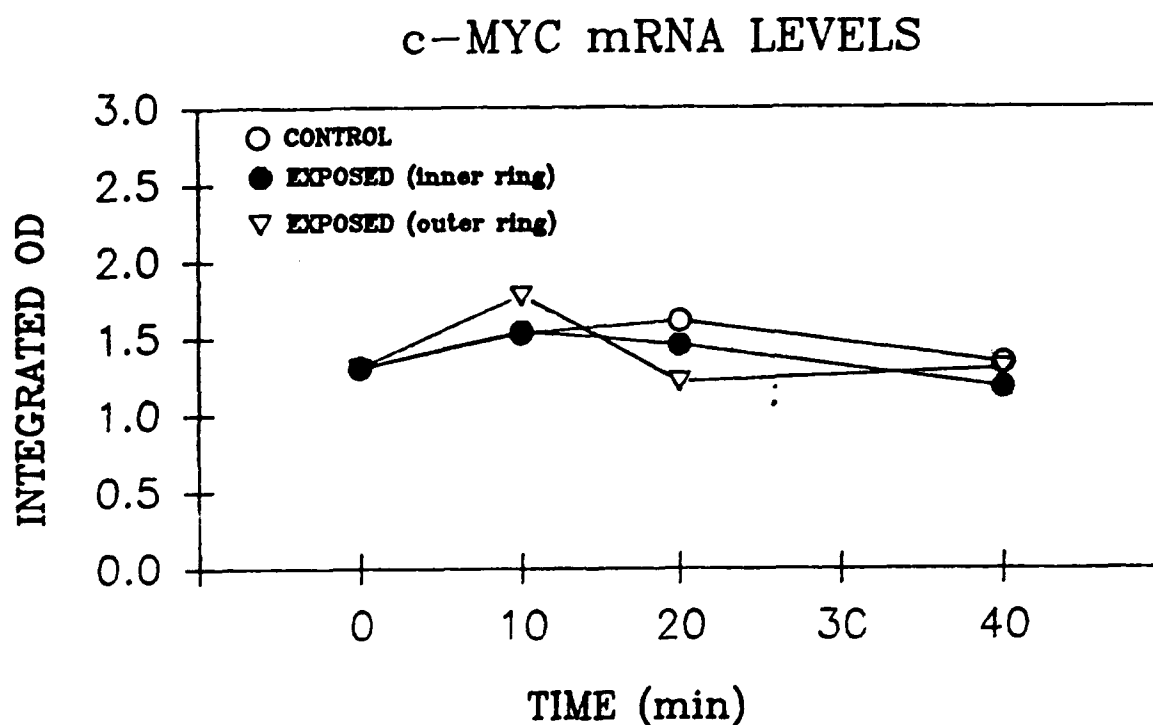
Fig. 2.8. As for Figure 2.7 except a solenoid exposure system was employed, with exposed cultures in inner and outer chambers of a concentric chamber culture dish, and RNA samples were Northern blotted prior to autoradiography. Samples were probed for c-myc mRNA. The exposure level was 100 µT. No enhancement of c-myc mRNA steady state levels were produced by ELF exposure.

47
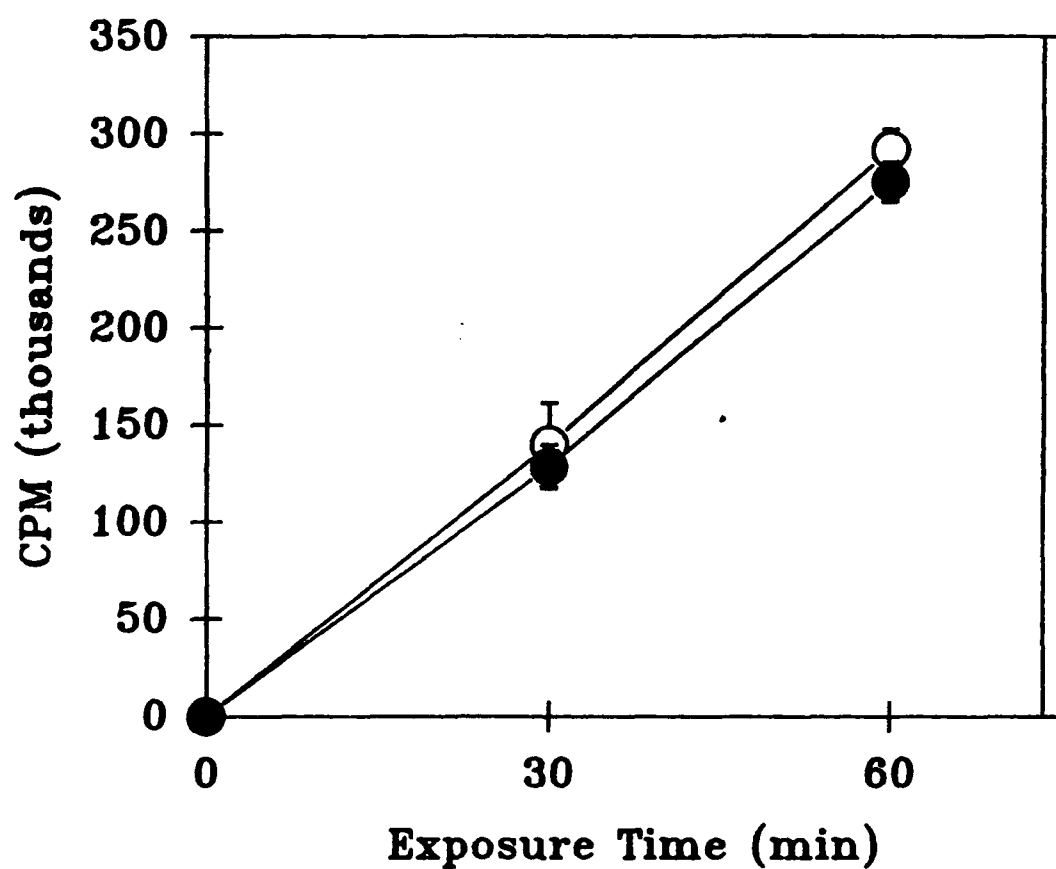
Fig. 2.9. Incorporation of tritiated uridine into total cellular RNA of HL-60 cells for control and 60-Hz field-exposed cultures. A 1.0 mT magnetic field was applied using the solenoid system. Cells were in the outermost chamber of the concentric chamber dishes. With continuous labeling over exposure times of 30 or 60 min no differences in labeling were evident.
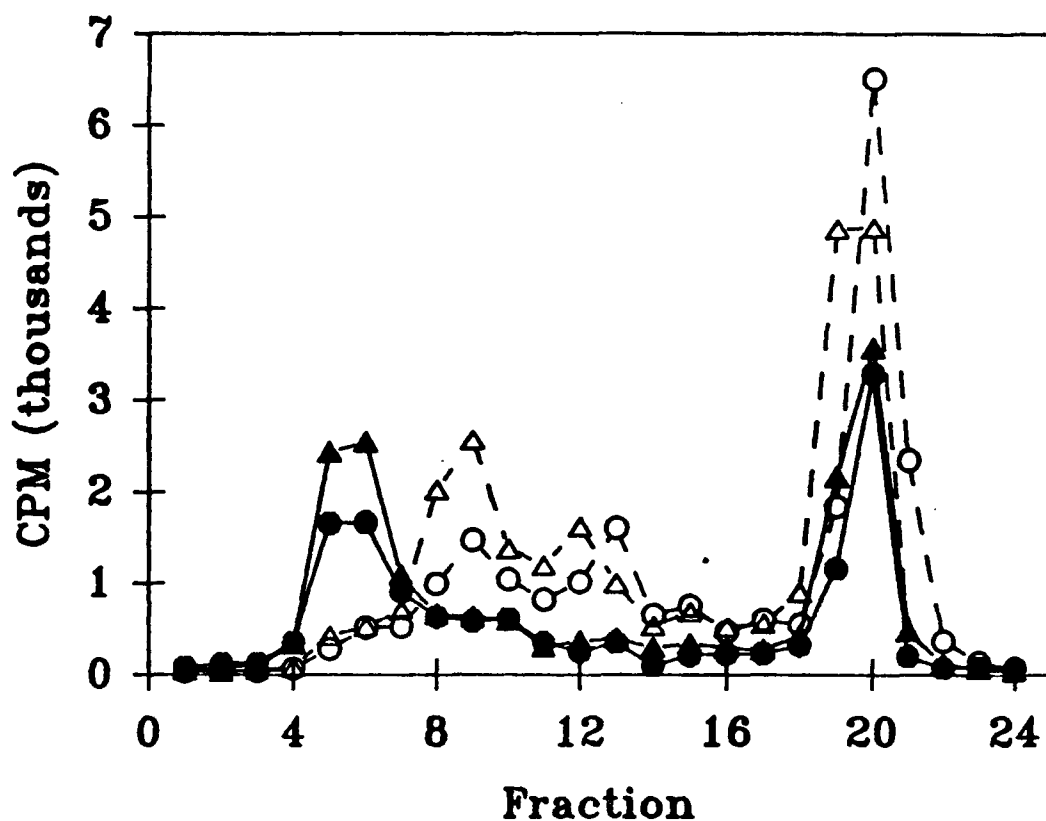
48

Fig. 2.10. Pulse-chase fractionation results of total cellular RNA from control and ELF exposed cells. HL60 cells were either untreated or exposed to 60-Hz, 1.0 mT fields for 90 minutes, and pulse labeled with ^{3}H-uridine as described in the text. After pulse-labeling the labeling medium was removed and the cultures were washed and chased with 1,000 X excess cold uridine for an additional 15 or 90 min. Solid triangles are exposed cells with a 15 min chase, open triangles are irradiated cells with a 90 min chase. Solid circles represent control cells with a 15 min chase, and open circles are control cells with a 90 min chase.

45S rRNA PROCESSING
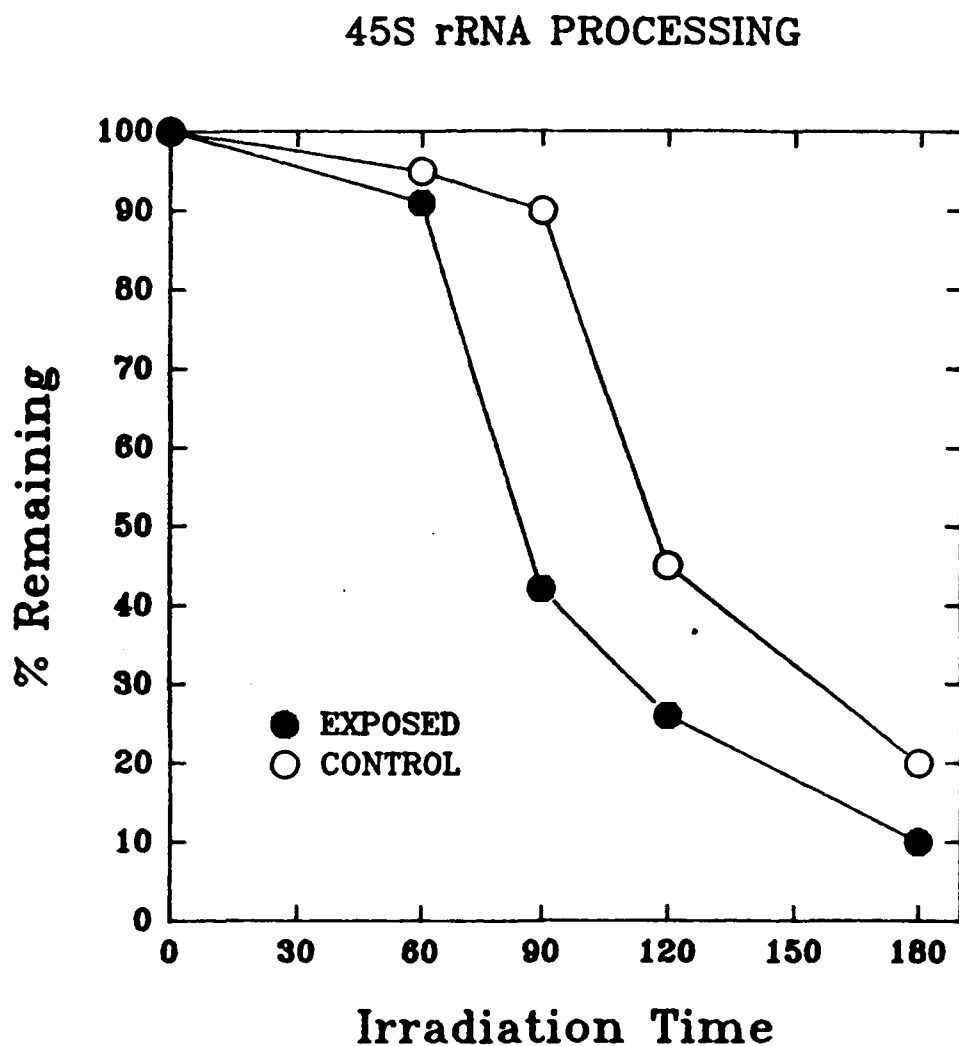
Fig. 2.11. Changes in the extent of labeling of the 45S rRNA precursor using pulse chase methods. RNA was labeled with tritiated uridine and then the label incorporated into the 45S peak was followed for different chase times. Cells exposed to a 1.0-mT, 60-Hz magnetic field in a solenoid device (cells were in the outermost chamber of the concentric dishes) displayed a faster rate of processing time for the 45S rRNA than did control cells.

2.7. REFERENCES

Byus, C. V., K. Kartun, S. Pieper and W. R. Adey (1988) Increased ornithine decarboxylase activity in cultured cells exposed to low energy modulated microwave fields and phorbol ester tumor promoters. Cancer Research, *48*:4222-4226.

Byus, C. V., R. L. Lundak, R. Fletcher and W. R. Adey (1984) Alterations in protein kinase activity following exposure of cultured human lymphocytes to modulated microwave fields. Bioelectromagnetics, *5*:341-352.

Byus, C. V., S. E. Pieper and W. R. Adey (1987) The effects of low-energy 60-Hz environmental fields upon the growth-related enzyme ornithine decarboxylase. Carcinogenesis (Lond.), *8*:1385-1389.

Byus, C. V. *et al.* (1990) Department of Energy, Contractors Review, Denver, Colorado, November 1990.

Chomczynski, P. and N. Sacchi (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162*:156-159.

Cleary, S. F., L-M. Liu and R. E. Merchant (1990) *In vitro* lymphocyte proliferation induced by radio-frequency electromagnetic radiation under isothermal conditions. Bioelectromagnetics, *11*:47-56.

Cossarizza, A., D. Monti, F. Bersani, M. Cantini, R. Cadossi, A. Sacchi and C. Franceschi (1989) Extremely low frequency pulsed electromagnetic fields increase cell proliferation in lymphocytes from young and aged subjects. Biochemical and Biophysical Research Communications, *160*:692-698.

Goodman, R. and A. S. Henderson (1987) Patterns of transcription and translation in cells exposed to EM fields: A review, in *Mechanistic Approaches to Interactions of Electric and Electromagnetic Fields with Living Systems*.

Goodman, R., C. A. L. Bassett and A. S. Henderson (1983) Pulsing electromagnetic fields induce cellular transcription. Science, *220*: 1283-1285

Goodman, R., J. A. and A. S. Henderson (1987) Transcriptional patterns in the X chromosome of *Sciara coprophila* following exposure to magnetic fields. Bioelectromagnetics *8*:1-7.

Goodman, R. and A. S. Henderson (1988) Exposure of salivary gland cells to low frequency electromagnetic fields results in alterations in polypeptide synthesis. Proc. Natl. Acad. Sci. USA, *88*:3928-3932.

Goodman, R, and A. S. Henderson (1990). Department of Energy Contractors Review. Denver, Colorado, November, 1990.

Goodman, R., L-X. Wei, J-C. Xu and A. S. Henderson (1989) Exposure of human cells to low-frequency electromagnetic fields results in quantitative changes in transcripts. Biochimica et Biophysica Acta, 1009:216-220.

Greene, J. J., W. J. Skowronski, J. M. Mullins, R. M. Nardone, L. M. Penafiel and R. Meister (1991) Delineation of electric and magnetic field effects of extremely low frequency electromagnetic radiation on transcription. Biochem. Biophys. Res. Comm., in press.

Heby, O. and L. Persson (1990) Molecular genetics of polyamine synthesis in eukaryotic cells. Trends in Biochemical Sciences, 15:153-158.

Luben, R. A., C. D. Cain, M. C. Chen, D. M. Rosen and W. R. Adey (1982) Effects of electromagnetic stimuli on bone and bone cells in vitro: Inhibition of responses to parathyroid hormone by low energy fields. Proc. Natl. Acad. Sci. USA, 79:4180-4184.

Luben, R. A. and C. D. Cain (1984) Use of bone cell hormone response systems to investigate bioelectromagnetic effects on membranes in vitro. In Nonlinear Electrodynamics in Biological Systems, 23-34.

Lyle, D. B., P. Schecter, W. R. Adey and R. L. Lundak (1983) Suppression of T lymphocyte cytotoxicity following exposure to sinusoidally amplitude-modulated fields. Bioelectromagnetics, 4:281-292.

McConlogue, L., M. Gupta, L. Wu and P. Coffino (1984) Molecular cloning and expression of the mouse ornithine decarboxylase gene. Proc. Natl. Acad. Sci. USA, 81:540-544.

Pegg, A. E. (1988) Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. Cancer Res., 48:759-774.

Rodemann, H. P., K. Bayreuther and G. Pfleiderer (1989) The differentiation of normal and transformed human fibroblasts in vitro is influenced by electromagnetic fields. Exptl. Cell Res., 182:610-621.

Ross, S. M. (1990) Combined DC and ELF magnetic fields can alter cell proliferation. Bioelectromagnetics, 11:27-36.

Seely, J. E. and A. E. Pegg (1983) Ornithine Decarboxylase (mouse kidney). Methods in Enzymology, 94:158-161.

Weaver, J. C. and R. D. Astumian (1990) The response of living cells to very weak electric fields--the thermal noise limit. Science, 247:459-462.

Walleczek, J. and R. P. Liburdy (1990) Nonthermal 60-Hz sinusoidal magnetic-field exposure enhances Ca-45(2+) uptake in rat thymocytes--dependence on mitogen activation. FEBS Letters, *271*:157-160.

Zimmerman, S., A. M. Zimmerman, W. D. Winters and I. L. Cameron (1990) Influence of 60-Hz magnetic fields on sea urchin development. Bioelectromagnetics, *11*:37-46.

CHAPTER 3

DESIGN AND EVALUATION OF MICROWAVE AND ELF EXPOSURE SYSTEMS

3.1. MICROWAVE EXPOSURE OF CELLS IN SUSPENSION

Two microwave exposure systems were used during this past year, both of them using Crawford cells and operating at 915 MHz. Exposure with microwaves modulated by low frequency sinusoids was emphasized over exposure with CW. This was done on the basis of results in the literature and findings by this group indicating the increased sensitivity of the cell to modulated microwaves.

One of the Crawford cell systems was described in a previous annual report. This system used a modified IFI CC110 Crawford cell which could accommodate four 25 cm^2, 50 ml tissue culture flasks on a shelf perpendicular to the direction of wave propagation with two flasks at either side of the center conductor. The second system, installed this year, makes use of an IFI BC110 unit, specifically designed for experimentation with biological samples. This Crawford cell is smaller than the one used in a previous setup and, as a consequence, it requires the use of smaller containers. 25 cm^2, 30 ml tissue culture flasks fabricated by Nunc are adequate for this purpose. This Crawford cell has two shelves which can accommodate a single flask on either side of the center conductor. A total of four flasks can be exposed at one time; however, only a single shelf was used to insure exposure of the samples to the same power level.

3.1.1 SAR MEASUREMENTS IN CRAWFORD CELL BC110

Crawford cells are generally chosen for exposure of biological samples since they allow uniform E-field exposure under special conditions (Bassen, 1982). Ideally the sample should be positioned perpendicular to the direction of wave propagation and parallel to the electric field. Additionally, it should occupy no more than the center one-third of the distance between the center conductor and the outer plate. Because of constraints imposed by commercially available sizes of tissue culture flasks, the ideal exposure conditions are not met with either Crawford cell currently in use. With the first Crawford cell (IFI model CC110), 25 cm^2, 50 ml tissue culture flasks are being used. Two flasks are placed on each side of the center conductor occupying about 72% of the available width. The second Crawford cell (IFI model BC110) is being used with 25 cm^2, 30 ml tissue culture flasks. One flask is placed on each side of the center conductor occupying about 98% of the available width. Since these exposure configurations are not ideal, it was decided to make SAR measurements to determine the electric field distribution.

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The SAR within a sample placed in the Crawford cell depends on the type of container used, the volume of nutrient medium bathing the cultured cells, and the number of containers concurrently exposed. The SAR may be calculated from the excess power dissipation over that measured with empty containers. However, this method is subject to relatively large errors since the presence of the aqueous liquid medium causes redistribution of the electric field in the Crawford cell. More representative results may be obtained by measuring the rate of increase of temperature per unit time in response to a high power input signal.

SAR measurements were made in the IFI model BC110 Crawford cell using 25 cm², 30-ml tissue culture flasks. Two flasks were filled with 5 ml of cell nutrient medium and positioned at either side of the center conductor. The system was driven at 915 MHz using a HP 8657B signal generator and an AR 50W1000 traveling wave tube amplifier. A Weinschel double stub tuner was used to match the impedance of the loaded Crawford cell. A block diagram of the system is shown in Figure 3.1.

The rate of temperature increase was measured using a Luxtron model 3000 fiberoptic thermometer with 2 MPM 4 sensor probes. Seven of these sensors were positioned inside each flask, at its base, through small holes made at the top of the flask. The sensors were secured in place with adhesive tape (Figure 3.2). In this manner measurements were made at five levels along the base of each flask for a total of 35 points. Each run was carried out with an input power of 57-60 watts for a period of approximately 60 seconds. This measuring interval was necessary to achieve at least a 2 to 4 °C temperature rise in the seven points probed at a given time. The rate of temperature decrease after the power was turned off was negligible over a period of 10 to 20 seconds when the temperature was 2 to 4 °C above the starting temperature. To minimize the error due to heat loss the SAR was determined using the temperature vs. time data for the first 10 seconds after the power was turned on. This kept the temperature increment below 2°C in all cases. These measurements were conducted at WRAIR in collaboration with Mr. Chuck Gambrill whose assistance we acknowledge with gratitude.

The SAR was calculated using the well known relation $SAR = C_p \, \Delta T/\Delta t$, where C_p is the specific heat of the exposed medium, and $\Delta T/\Delta t$ is the rate of temperature increase per unit time. For these calculations, C_p was assumed to be 3.95 J/°C/g. Figures 3.3(a) and (b) show the SAR distribution for flasks positioned to the left, and to the right of the center conductor, respectively. These results indicate that, for this configuration, the electric field in each chamber is similar to that present in a rectangular waveguide of equivalent dimensions. Hence, the modes present in the Crawford cell under these conditions are no longer TEM modes.

A comparison of Figures 3.3(a) and (b) shows that the SAR is generally larger in the right hand side chamber. Careful measurements of the width of each chamber indicated that the width of the left hand side chamber is about 4% larger than that of the right hand side chamber. Accordingly, the peak SAR in the left hand side chamber is about 30% smaller than that in the right hand side chamber. This points out the need for exact symmetry to either side of the center conductor of a Crawford cell in order to obtain equivalent E-field distributions.

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3.2. MEASUREMENTS OF INDUCED ELECTRIC FIELDS WITHIN SAMPLES EXPOSED TO ELF MAGNETIC FIELDS

A significant effort was expended during the past year to characterize induced electric fields within biological samples exposed to ELF magnetic fields. As a result of this work, we have gained considerable experience about the problems associated with this type of measurement. Using this background, in collaboration with Howard Bassen of FDA, we have been developing guidelines to help experimentalists select appropriate exposure configurations for which the electric and magnetic field components are well characterized.

The comparison of measured and calculated values of the induced electric field was important in establishing the above mentioned guidelines. On the basis of this comparison it was concluded that the induced electric field for certain commonly used configurations could be calculated from theory and tabulated to eliminate computational complexity. Previously reported measurements of induced electric fields, performed to support this work, were carried out using a dipole probe whose leads were encased in a glass tube. When making measurements along boundaries parallel to the stem of the probe, the proximity of the dipole elements to the boundary was limited by the radius of the glass tube. Therefore, to compare measured and theoretical values it was necessary to make the theoretical calculations at the appropriate distance away from the interface. The determination of the electric field along the boundary corresponding to the base of the dish is of particular interest since the cells generally lie on this boundary after settling. In order to make measurements as close to the boundary as possible, a section of the glass tube was cut off along its long axis (Figure 3.4). Additionally, the shunting resistor which was previously placed next to the dipole elements, was positioned close to the amplifier.

Measurements using the new probe were conducted with circular and rectangular containers oriented with their bases parallel to the direction of the magnetic field. Cut out containers standing on their side walls were used for both cases. The containers were filled with a 0.15 N NaCl solution. The results of these measurements are shown in Figures 3.5 and 3.6. Calculated values using McLeod's equations (McLeod, Pilla and Sampsell, 1983) are also plotted in these figures. These calculations were made assuming that the dipole elements were positioned 1 mm away from the boundary. In both cases good agreement between measured and theoretical values is observed, corroborating previous observations.

3.3. EXPOSURE OF BIOLOGICAL SAMPLES TO ELF INCOHERENT EM SIGNALS

3.3.1. DIRECT ELF EXPOSURE

Experimental evidence of detrimental effects on the development of chick embryos resulting from exposure to ELF fields have been previously reported in the literature and also observed in our laboratories. These results were obtained by reproducing the experimental conditions of the "Henhouse" experiment. The ELF field was produced by a pulse train consisting of narrow (0.5 ms) pulses with a peak voltage of 1.23 V and a fixed period $\tau = 10$

ms. It has been suggested in the literature that the observed effects depend on the coherence time of the exposure signal. The coherence time refers to the period during which the signal retains phase continuity. The effects of changing the coherence time of the ELF signal has been extensively studied during this past year and is presented in Chapter 2 of this report. To reduce the coherence of the Henhouse pulse train, a delay period of random duration $\Delta\tau$ between 0 and 0.5 τ was introduced at fixed intervals of duration $T = 100$ ms. Since the triggering signal is computer controlled, any combination of randomization times is possible limited by the response time of the components of the system.

3.3.2 MODULATED MICROWAVE EXPOSURE

A similar arrangement was set up for experiments in which the exogenous field was a 915-Hz microwave signal sinusoidally modulated at ELF frequencies. The Crawford cell exposure system was used to observe the effect on certain biological markers of exposure to these fields. To investigate the "requirement" of a coherent impressed field, the modulation signal was set to alternate between two frequencies, 55 Hz and 65 Hz. Each frequency was set to be on for a fixed period T plus a time interval $\Delta\tau$ of random duration between 0 and 50 ms. The period T determines the number of cycles during which coherence is maintained and is the experimental parameter to be varied to determine the coherence time needed to obtain a given biological effect.

3.4. MEASUREMENT OF THE DC CONDUCTIVITY OF EGG WHITE AND EGG YOLK

Extensive research has been carried out during this past year on possible effects of very low level ELF fields on the development of chick embryos. This work consists of exposing newly fertilized eggs to ELF fields and subsequently analyzing for morphological or developmental abnormalities of the young embryo which is physically located on the periphery of the egg yolk. The egg white and the egg yolk have different compositions and are expected to have different conductivities as well. It was found in our previous work that the distribution of the induced electric field within a conducting sample bounded by an insulating layer depends has its center of symmetry within the sample and is independent of the distribution in the external medium. Moreover, if samples of different conductivities are present the E-field is likely to be distorted due to charge buildup at the interface between samples. This effect is expected to be more pronounced at the interface itself--in this case near the embryo. Because of these antecedents it was of interest to make conductivity measurements within both the egg white and the egg yolk. Non-fertilized eggs were used for this and other measurements reported here.

Conductivity measurements were carried out using a home-made conductivity probe. This probe was constructed similarly to the E-field probes, i.e, made of Belden wire #8640 held within a glass tube. A 1000-ohm shunting resistor was used at the junction between the probe leads and the probe elements. The elements consisted of two platinum gauge 24 wires coated with

platinum black running parallel to each other and separated by about 2 mm. This probe was used in conjunction with the HP 4194A automatic Impedance/Gain Phase Analyzer.

The probe was calibrated using various NaCl solutions of known conductivity. A calibration constant was obtained from these measurements which allows direct determination of the sample conductivity from the measurement of the sample conductance. Table 3.1 shows the results of measurements made in the range between 100 Hz and 400 Hz. For neither the white nor the yolk was any significant variation with frequency observed. The conductivity of the egg white was found to be approximately 2.6 times greater than that of the yolk.

Table 3.1. Electrical conductivity of egg white and egg yolk as a function of frequency.

Frequency (Hz)	Conductivity (S/m)	
	Egg white	Egg yolk
100	0.53347	0.20382
150	0.53786	0.20507
200	0.53812	0.20552
250	0.54272	0.20602
300	0.54419	0.20675
350	0.54530	0.20709
400	0.54633	0.20717

3.5. MEASUREMENT OF INDUCED ELECTRIC FIELDS IN TWO LAYER SYSTEMS

Time varying magnetic fields within a human subject generate electric fields and currents determined by morphology and composition. The dielectric properties of biological tissues can vary widely depending on composition and water content. For instance, the conductivity of bone is 0.013 S/m while that of whole blood is 0.6 S/m (Foster and Schwan, 1986). At interfaces between tissues of different conductivity, the magnetic field causes a buildup of charge which contributes to the magnitude and orientation of the induced electric field. The net effect of the induced interfacial polarization depends on the extent of the conductivity difference between

neighboring tissues. Even though the overall effect may not be very large in most cases, it is important to recognize its presence when studying biological effects of ELF fields.

A case of particular interest in our work is that of *in vitro* ELF exposure of biological cell suspensions. When the suspended cells settle at the bottom of the container, the sample consist of a two-layer composite. The bottom layer consists primarily of densely-packed cells while the top layer is made up of the suspending nutrient medium. One commonly used exposure configuration employs a Helmholz coil within which the cell suspension is placed with the base of the flask parallel to the direction of the magnetic field. The equivalent two layer problem has been solved numerically for a container of rectangular geometry (Wang, 1991).

To provide experimental results against which to test the numerical calculations, measurements were made using a two layer composite sample placed in a 25-cm^2, 50-ml tissue culture flask (Corning #25100-25) such that each layer was parallel to the base of the flask. The bottom layer consisted a sodium chloride solution with conductivity σ_1 in a 1% agarose gel. The top layer consisted of an aqueous sodium chloride solution of conductivity σ_2. The flask was positioned within a 22-cm diameter Helmholz coils with its base parallel to direction of the magnetic field. A 400-Hz, 100-μT magnetic field was used for the exposure.

Measurements of the induced electric field were made using a 4-mm electric dipole probe. This probe was built using a shielded twisted pair with platinum dipole elements coated with platinum black. The probe was positioned in the flask such that the twisted pair leads were parallel to the direction of the magnetic field up to the input to the amplifier. This was done to minimize extraneous pick up. To prevent spillage of the sample though the probe placement hole, the probe was positioned in place and the hole sealed with putty prior to pouring the sample into the container (Figure 3.11). The side opposite the base of the flask was cut out to facilitate introduction of the sample. The agarose layer was poured first into the flask while still in the liquid state and allowed to gel before pouring the upper liquid layer.

Proper use of the electric dipole probe required determination of the background voltage pick up. This background voltage must be subtracted from the probe output reading to determine the electric field using the probe constant. The probe background was obtained by determining the probe output voltage at a point within the medium used for calibration where the electric field was expected to be zero. This can be easily achieved when the probe is positioned anywhere along the center vertical axis of the surface perpendicular to the direction of the magnetic field. In this case, the background is determined by adding to the empty container enough liquid of the desired conductivity such that the probe is at the geometrical center of the plane where the field is induced. The probe voltage at this position is the background. This measurement was carried out with the solution of conductivity σ_2, that is the solution within which E-field measurements with the two layer were made. This was necessary since the probe calibration may vary depending on the conductivity of the solution. Because of this calibration procedure, measurements in the gel phase were not practical.

A potential source of errors in the measurement results from ionic diffusion between layers. While diffusion can not be eliminated it can be minimized by rapidly pouring the liquid phase and immediately measuring the output of the electric field probe. In general this can be done within 15 to 30 seconds from the time the liquid sample is poured.

Measurements were conducted with the probe positioned at a height of approximately 7 mm from the base of the container. The gel phase was poured to a height of approximately 3 mm while the liquid phase reached a height of approximately 8 mm over the gel as shown in Fig. 3.12. Two conditions were considered, one in which σ_1 was a factor of ten times lower than σ_2 and another one in which the values of σ were reversed. The results are summarized in Table 3.2. The uncertainty in the measured values represent the estimated error in the determination of the probe constant. The numerically calculated values listed in the table were determined assuming that all parameters are precisely known. However, in our measurements, the estimated uncertainties for the for the liquid heights and the probe position were of the order of $\pm$ 0.5 mm. If these uncertainties are introduced in the numerical calculation, the resulting electric field varies in the range $\pm$ 1.5 V/m. With these considerations, it can be said that the agreement between the numerical calculations and the experimental measurements is quite good. This is shown graphically in Figure 3.13 where the effect of the presence of the interface is shown with respect to the homogeneous case and McLeod's results.

Table 3.2. Comparison of calculated and measured electric fields within the liquid phase at the location of the dipole probe shown in Fig. 3.12.

σ_1 (S/m)	σ_2 (S/m)	E Calculated (mV/m)	E Measured (mV/m)
0.015	0.15	0.434	0.682 $\pm$1
0.15	0.015	7.87	7.86 $\pm$1

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3.6. FIGURES
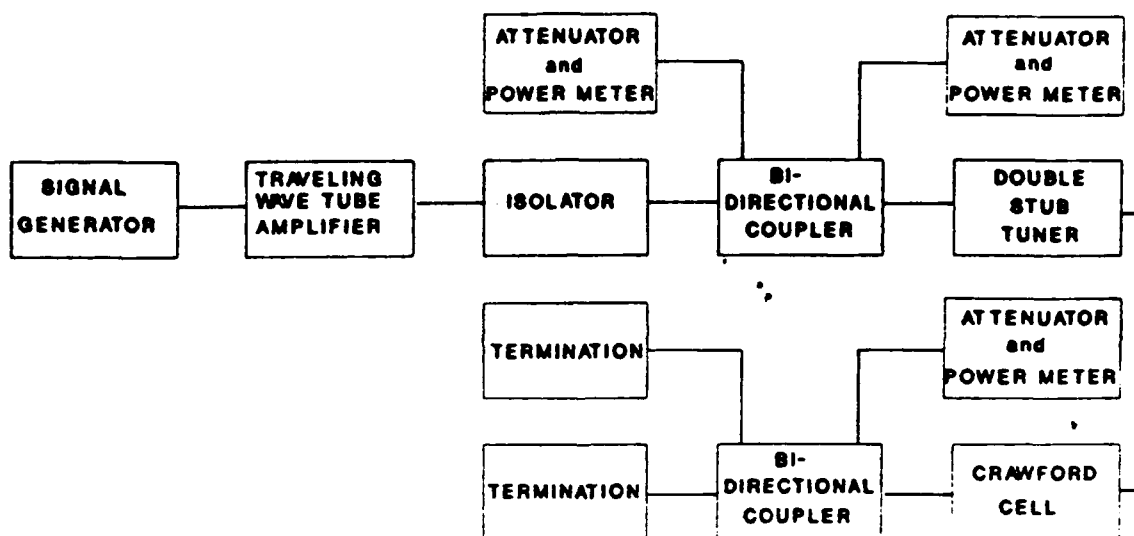
Fig. 3.1 Block diagram of measuring system to determine SAR in an IFI-BC110 Crawford cell.

TEMPERATURE PROBE
INSERTION HOLES

ADHESIVE TAPE

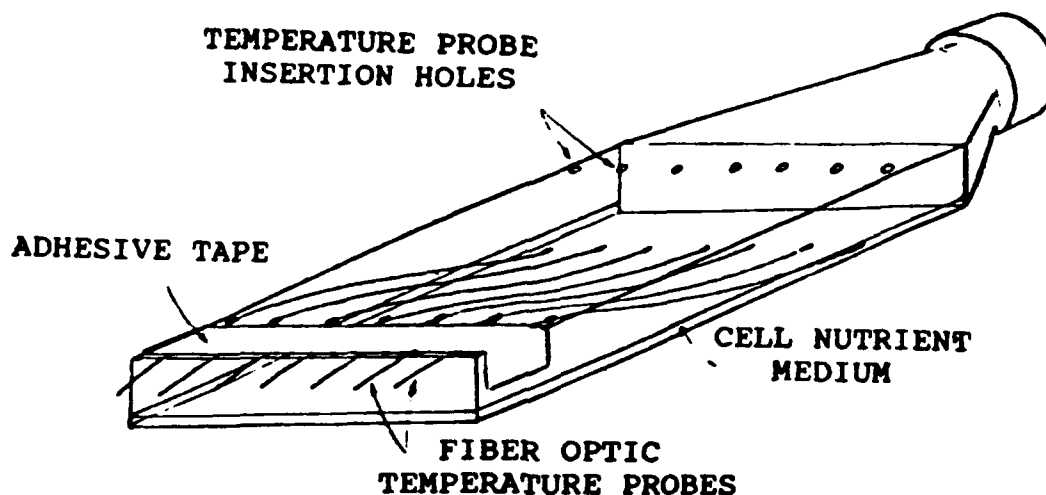CELL NUTRIENT
MEDIUM

FIBER OPTIC
TEMPERATURE PROBES

Fig. 3.2. Diagram of perforated tissue culture flask showing positioning of fiber optic sensors. The sensors were placed in either set of holes depending on where the measurement was being made.
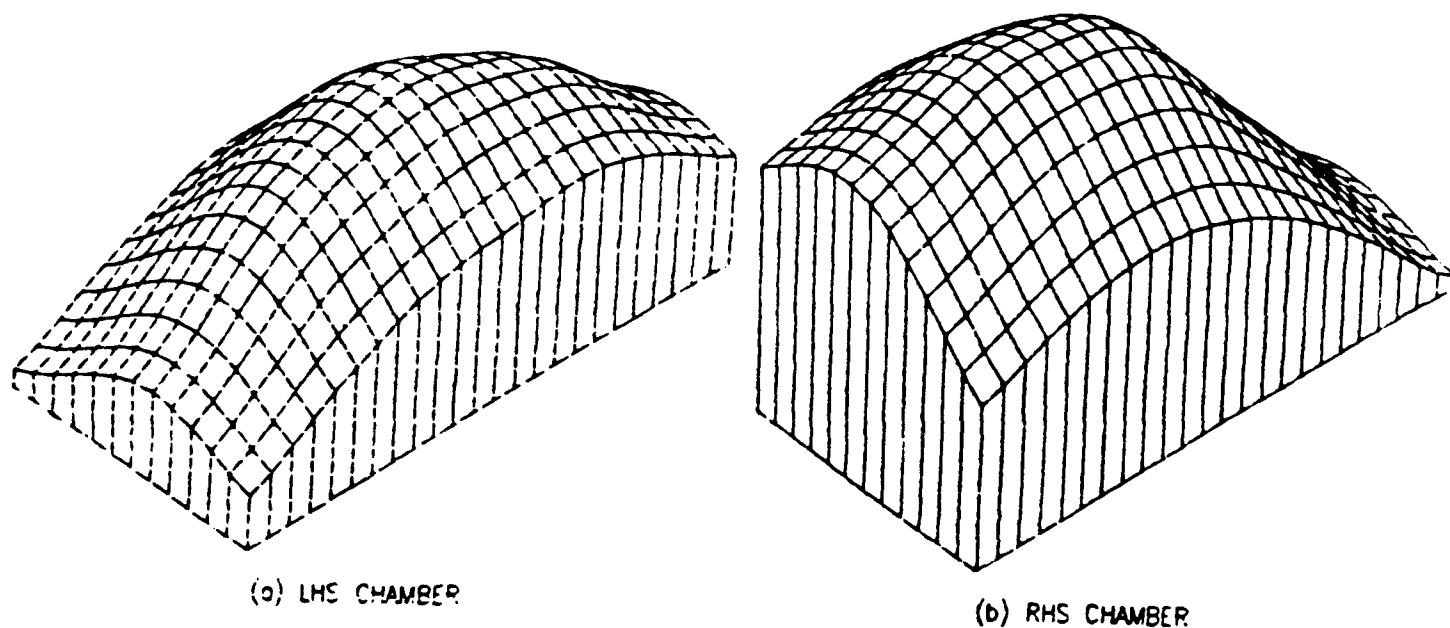

(a) LHS CHAMBER

(b) RHS CHAMBER

Fig. 3.3. SAR distribution in tissue culture flasks filled with 5 ml of nutrient medium and placed in (a) the left side chamber and (b) the right side chamber of an IFI-BC110 Crawford cell.

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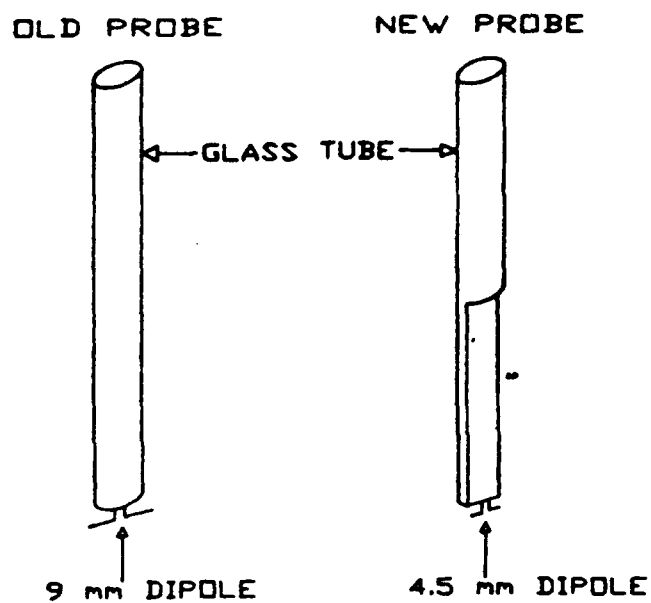
Fig. 3.4. Sketches of the electric field probes showing the modification made to allow measurements to be made close to a boundary.
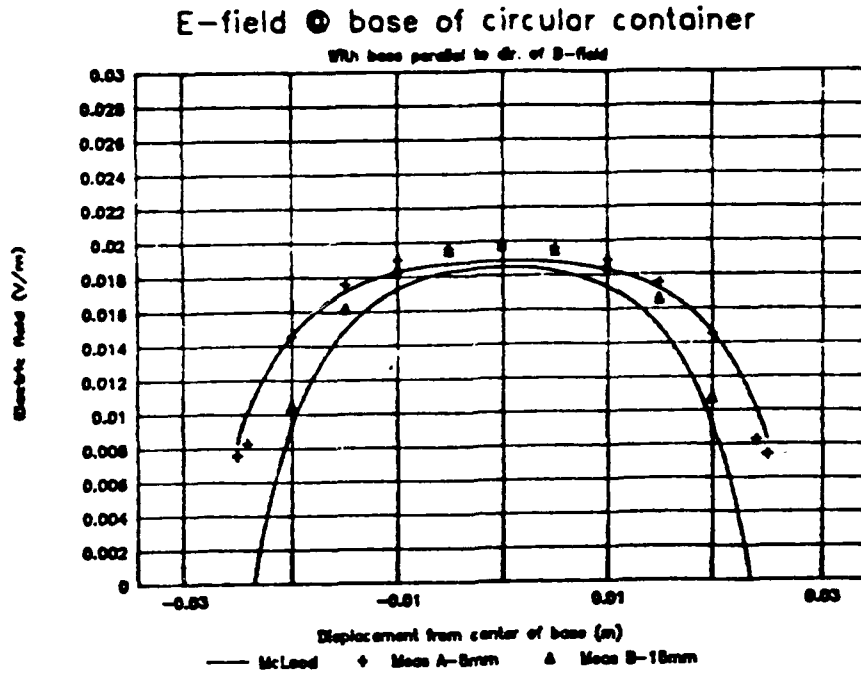
Fig. 3.5. Measured and calculated E-fields along parallel chords at the base of a circular dish. Chords A and B are 5mm and 15mm respectively from the center of the container.
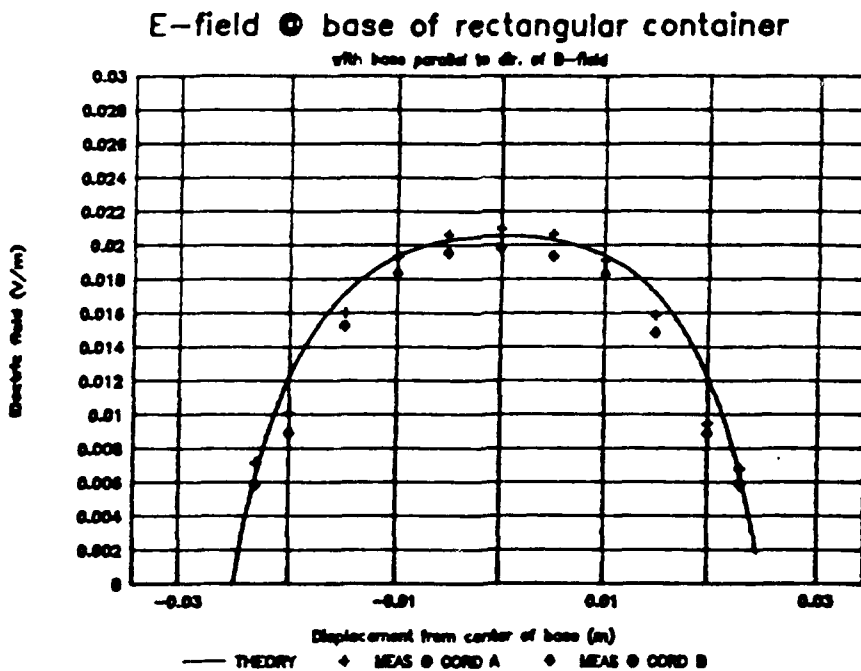
Fig. 3.6. Measured and calculated E-fields along chords perpendicular to the direction of the B-field at the base of a rectangular dish. Chords A and B are separated by 5mm.
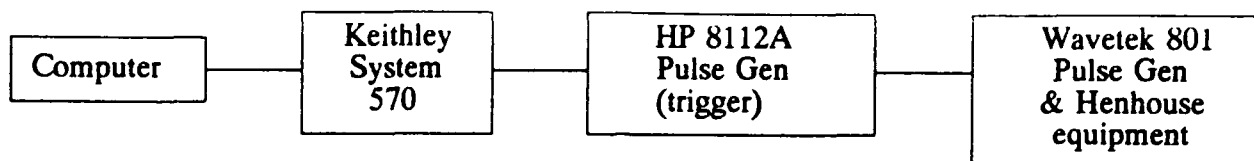
64

Fig. 3.7. Block diagram of instrumentation to produce an incoherent pulse train for ELF exposure experiments.
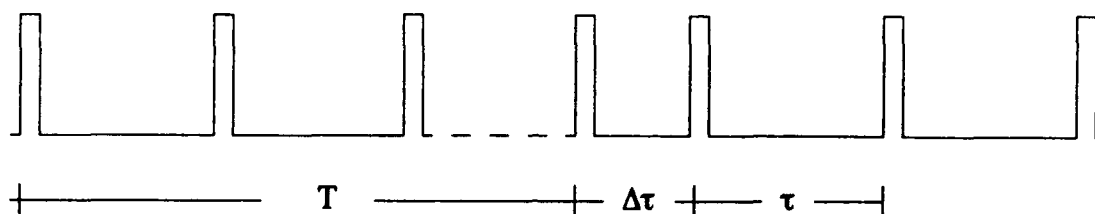
Fig. 3.8. Timing diagram of pulse train for ELF experiments with chick embryos.
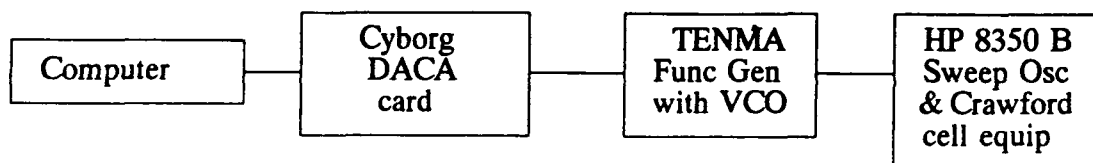
Fig. 3.9. Block diagram of instrumentation to produce an incoherently modulated microwave signal for Crawford cell microwave irradiation experiments.
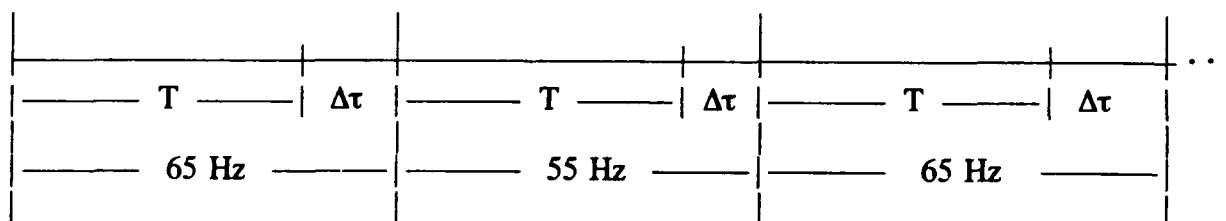

Fig. 3.10. Timing diagram for ELF signal used to modulate the microwave field for exposure of cells in suspension using the Crawford cell.
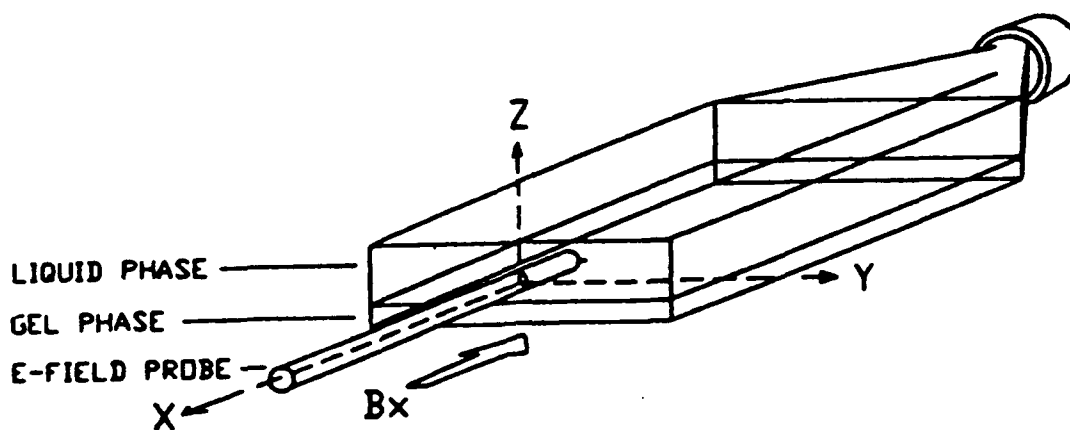
65

Fig. 3.11. Tissue culture flask with a liquid and a gel phase showing positioning of the dipole probe for electric field measurements.
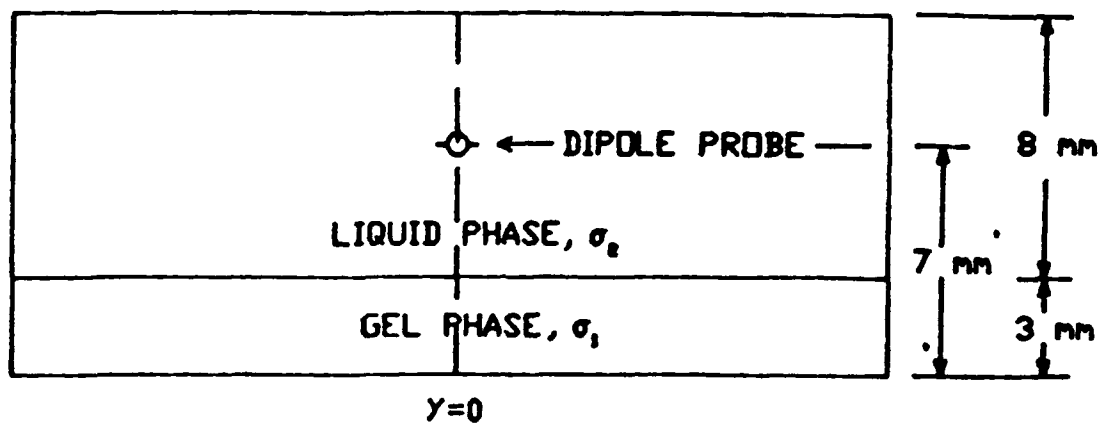

Fig. 3.12. Detail of two layer system showing the height of each layer and the position at which E-field measurements were made.
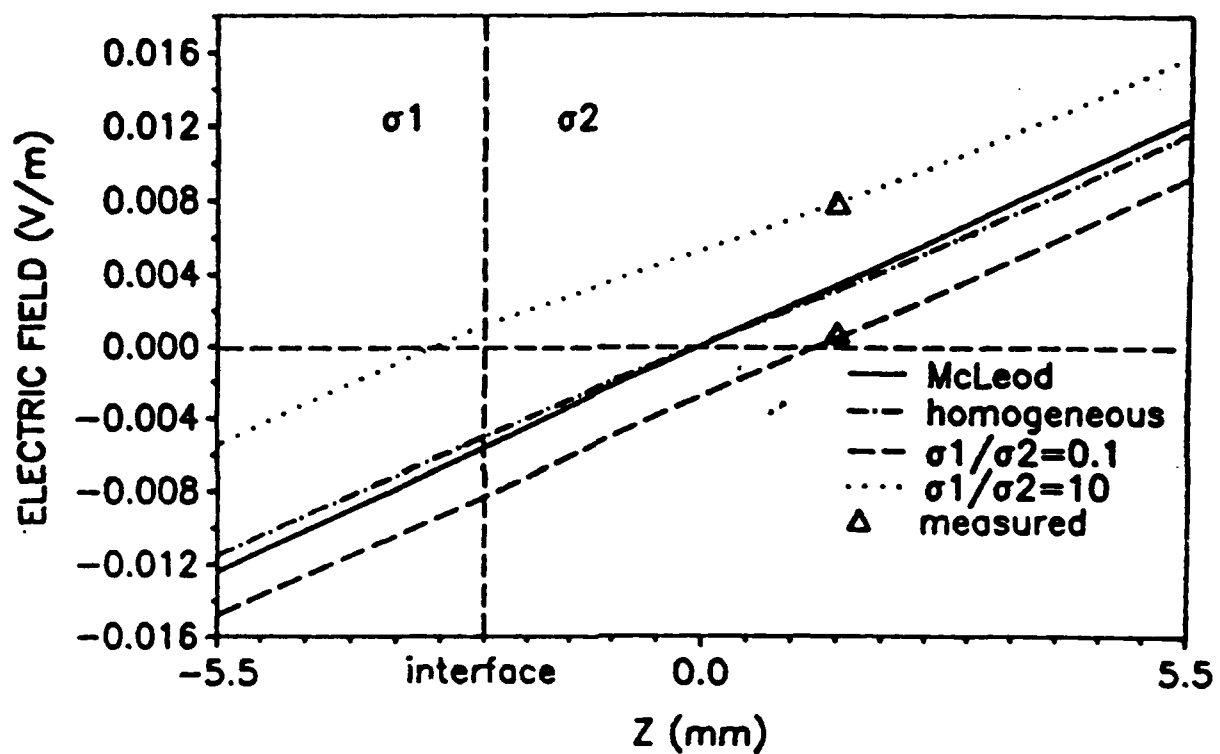
66

Fig. 3.13. Electric field distribution at y=0 along the z-axis for $\sigma_1/\sigma_2 = 0.1$ and $\sigma_1/\sigma_2 = 10$. The E-field for both the homogeneous and inhomogeneous cases are shown for comparison.

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3.7. REFERENCES

Bassen, H. (1982) Pitfalls of certain plane wave exposures of biological specimens. Poster presentation at the 1982 Annual Meeting of the Bioelectromagnetics Society.

Foster K. R. and H. P. Schwan (1986) in *CRC Handbook of Biological Effects of Electromagnetic fields*, 27-96.

McLeod B. R., A. A. Pilla and M. W. Sampsell (1983) Electromagnetic fields induced by Helmholz coils inside saline filled boundaries. Bioelectromagnetics, *4*:327-370.

Wang, W. (1990) private communication.

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